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THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 10 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years; and in addition there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XI. The accounts of the Treasurer shall be audited annually by a certified public accountant.

XII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1943.

The accounts have been audited by Messrs. Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and inspection of it by members of the Corporation will be welcomed.

The principal summaries of their report—The Balance Sheet, Statement of Income and Expense, and Current Surplus Account—are appended hereto as Exhibits A, B and C.

The following are some general statements and observations based on the detailed reports:

I. Assets

1. Endowment Assets

As of December 31, 1943, the total of all the Endowment Assets was $1,003,708.63, a loss of $68,282.27 from the total for the preceding year. This substantial loss was due almost entirely to the sales of three New York City real estate holdings on which the Laboratory Trust Funds with the Central Hanover Bank and Trust Company had first mortgage participations. The three properties sold were a 15-story office building at 89 Madison Avenue, a 7-story loft building at 25 Spruce

Street, and an apartment house at 127 West 79th Street. The Laboratory's total investment in the three mortgages was $98,315.24; from the foreclosures and sales the Laboratory received $16,827.70 cash and new mortgages for $16,251.81; the total capital loss was thus $65,235.73.

During 1943 the *market* values of the Laboratory's securities, except for the mortgage and real estate participations, made a substantial gain. As of December 31, the totals of government and corporate bonds, common and preferred stock holdings were $754,904.23 book or cost value, with a market value of $750,286.38.

The total of mortgage and real estate participations was $219,719.95 book value, approximately 22 per cent of the total. There are no market quotations for these securities, but in view of the loss of over 60 per cent of the cost of the three real estate holdings sold last year, the generally deteriorating position of New York City property, and the difficulties now besetting some of the other 16 mortgages in which the Laboratory has participations (one of them has already been foreclosed) it would be unwise to estimate their actual value at much more than 50 per cent of cost, or about $110,000. The actual value of the Endowment Funds including uninvested cash of $29,084.45, would thus be approximately $889,370.83.

2. Plant Assets

The total of Plant Assets (excluding the Gansett and Devil's Lane Tracts) was $1,341,425.88 after deduction of $633,901.42 accumulated Depreciation Reserve, a decrease of $16,336.11. During the year, depreciation charges deducted amounted to $26,969.11. The Reserve Fund was increased by $8,998.71 cash transferred from current income, representing profits on sale of Gansett lots, and a portion of the dividends received on the General Biological Supply House and Crane Company stocks.

3. Current Assets

Current Assets including cash, inventories and investments not in the Endowment Funds, amounted to $194,111.69, an increase of $29,442.67. Current Liabilities were only $3,251.25. After the setting up of a special reserve of $2,358.34 for repairs and replacements on the properties leased by the Navy, the total of Current Surplus was $188,502.10, an increase of $28,829.33 over 1942.

II. Income and Expenditures.

Total Income was $159,296.94, an increase of $13,227.18 over the preceding year. Total Expenses, including the $26,969.11 added to Depreciation Reserves, were only $139,973.27 compared with $159,296.94, so that for 1943 there was an actual net operating surplus of $19,323.67 in gratifying contrast with the deficit of $17,211.93 for 1942.

This excess of income over expenditures is due to a number of factors. One was the $20,150.00 received or accrued as rentals from the United States Navy. Not all of this total was a clear increase, of course, as normal income from the leased properties was eliminated, but at least a portion of it represented increased income which will not again be available. Income from the General and Library Endowment Funds reversed the recent downward trend and rose slightly to $36,610.98. More substantial gains over 1942 were shown in the income from the Supply Department which gave a profit of $14,436.21 compared with the 1942 deficit of $1,699.05 on only a slightly increased volume of sales, and in the dividends from the General Biological Supply House stock which were $18,796.00 as against $10,922.00.

Even more important than the increases in income were the careful reductions in expenditures made by the Laboratory Administration. Maintenance expenses were still further cut in nearly every department, and purchases and improvements were kept at a minimum to an extent that would be impossible under normal operating conditions.

Both income and expense totals must therefore be regarded as abnormal because of the special circumstances prevailing in the year 1943.

EXHIBIT A

Marine Biological Laboratory Balance Sheet, December 31, 1943

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee		$ 993,773.34	
Securities and Cash in Minor Funds		9,935.29	
			$1,003,708.63
Plant Assets:			
Land	$ 111,425.38		
Buildings	1,322,348.21		
Equipment	185,890.98		
Library	321,626.42		
	$1,941,290.99		
Less Reserve for Depreciation	633,901.42	$1,307,389.57	
Cash in Reserve Fund		13,272.22	
Cash in Book Fund		20,764.09	
			$1,341,425.88
Current Assets:			
Cash		$ 23,312.61	
Accounts Receivable		12,217.19	
Inventories:			
Supply Department	$ 42,826.62		
Biological Bulletin	17,971.16		
		$ 60,797.78	
Investments:			
Devil's Lane Property	$ 45,967.76		
Gansett Property	2,694.70		
Stock in General Biological Supply House, Inc.	12,700.00		
Other Investment Stocks	17,770.00		
Retirement Fund	13,242.17		
		$ 92,374.63	
Prepaid Insurance		4,928.83	
Items in Suspense		480.65	
			$ 194,111.69
Total Assets			$2,539,246.20

Liabilities

Endowment Funds:			
Endowment Funds	$ 991,628.91		
Reserve for Amortization of Bond Premiums	2,144.43		
		$ 993,773.34	
Minor Funds		9,935.29	
			$1,003,708.63
Plant Liabilities and Surplus:			
Donations and Gifts		$1,172,564.04	
Other Investments in Plant from Gifts and Current Funds		168,861.84	
			$1,341,425.88
Current Liabilities and Surplus:			
Accounts Payable		$ 3,251.25	
Reserve for Repairs and Replacements		2,358.34	
Current Surplus (Exhibit C)		188,502.10	194,111.69
Total Liabilities			$2,539,246.20

EXHIBIT B

Marine Biological Laboratory Income and Expense, Year Ended December 31, 1943

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 29,310.84		$ 29,310.84
Library Fund		7,300.14		7,300.14
Instruction	$ 6,598.09	5,085.00	$ 1,513.09	
Research	4,488.30	8,662.50		4,174.20
Evening Lectures	25.00		25.00	
Biological Bulletin and Membership Dues	5,292.02	6,859.33		1,567.31
Supply Department	27,215.89	41,652.10		14,436.21
Mess	17,237.17	19,093.67		1,856.50
Dormitories	22,605.46	15,439.65	7,165.81	
(Interest and Depreciation charged to above 3 Department)	24,866.25			24,866.25
Dividends, General Biological Supply House, Inc.		18,796.00		18,796.00
Dividends, Crane Company		500.00		500.00
Rents:				
Bar Neck Property	717.77	3,600.00		2,882.23
Janitor House	29.17	360.00		330.83
Danchakoff Cottages	267.47	300.00		32.53
Lecture Hall and Botany building		999.96		999.96
Rooms in Laboratory, Special		245.00		245.00
Sale of Library Duplicates and Micro Flms		446.14		446.14
Microscope and Apparatus Rental		613.55		613.55
Sundry Income		33.06		33.06
Maintenance of Plant:				
Buildings and Grounds	19,529.79		19,529.79	
Apparatus Department	4,683.32		4,683.32	
Chemical Department	1,588.86		1,588.86	
Library Expense	6,463.00		6,463.00	
Workmen's Compensation Insurance	429.53		429.53	
Truck Expense	152.22		152.22	
Bay Shore Property	86.84		86.84	
Great Cedar Swamp	17.10		17.10	

General Expenses:				
Administration Expense	14,386.77		14,386.77	
Endowment Fund Trustee and Safe-keeping	1,014.45		1,014.45	
Bad Debts	720.29		720.29	
Special Repairs, Supply Dept. Stone Building	1,963.56		1,963.56	
Reserve for Repairs and Replacements, Buildings occupied by Navy	2,358.34		2,358.34	
Reserve for Depreciation	26,969.11		26,969.11	
	$139,973.27	$159,296.94	$ 89,067.08	$108,390.75
Excess of Income over Expense carried to Current Surplus	$ 19,323.67		$ 19,323.67	
	$159,296.94		$108,390.75	

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT, YEAR ENDED DECEMBER 31, 1943

Balance, January 1, 1943			$159,672.77
Add:			
Excess of Income over Expense		$19,323.67	
Gain on Gansett Lots Sold		279.41	
Bad Debt Recovered		.90	
Transfer of Biological Bulletins, held at Lancaster Press for Uncompleted Serial Exchanges, 1940, 1941, and 1942 from Plant Account, Library to Biological Bulletin Inventory		2,552.50	
Reserve for Depreciation charged to Plant Funds		26,969.11	
			49,125.59
			$208,798.36
Deduct:			
Payments from Current Funds during Year for Plant Assets:			
Buildings	$ 2,460.20		
Equipment	984.64		
Library	4,583.95		
	$ 8,028.79		
Less Received for Plant Assets Sold	140.00	$ 7,888.79	
Pensions Paid	3,460.00		
Loss on Retirement Fund Securities	181.48		
	$ 3,641.48		
Less Retirement Fund Income	232.72	3,408.76	
Transfers to Reserve Fund, Portion of Dividends of Prior Years from General Biological Supply House, Inc. and Crane Company	8,500.00		
Profit on Gansett Lots for 1941 and 1942	498.71	8,998.71	20,296.26
Balance, December 31, 1943			$188,502.10

Respectfully submitted,

DONALD M. BRODIE,
Treasurer

V. REPORT OF THE LIBRARIAN

The librarian's report for 1942 gave a detailed account of the readjustments that the library has gradually made to changes of the past few years in the budget, in the receipt of European and Asiatic current journals and in the available markets for purchases of "back sets." It is not necessary this year to comment on these adjustments except to state that definite effort has been made to renew exchange relationship with Russia, China and India and with the limited success that was anticipated. There should be some results to report for this in 1944–45, depending, of course, on the circumstances of the war.

The budget of $12,200 for 1943 was expended as follows: books, $314.33; serials, $1871.51; binding, $645.41; express, $64.29; supplies, $202.91; salaries, $7200.00; back sets, $815.77; sundries, $3.07; and insurance, $45.00; total, $11,162.29. The sale of duplicates brought in this year $214.03; and the income from the microfilm service amounted to $221.33, 99 orders having been filled.

From the "Carnegie Fund" $1745.92 was expended on two completed and 14 partially completed "back sets" and three books that are designated as "classics."

The Woods Hole Oceanographic Institution appropriated $1850.00 for 1943 and a balance of $70.11 remained from the 1942 budget. An expended sum of $1657.03 has been reported to the Director. A balance of $263.08 was carried on to the year 1944.

During 1943 the library received 645 current journals: 232 (10 new) by subscriptions to the Marine Biological Laboratory; 15 (none new) to the Woods Hole Oceanographic Institution; exchanges, 191 (two new) with the "Biological Bulletin" and 22 (six new) with the Woods Hole Oceanographic Institution publications; 181 as gifts to the former and four to the latter. The Marine Biological Laboratory acquired 113 books: 47 by purchase of the Marine Biological Laboratory (three "classics"); 11 by purchase of the Woods Role Oceanographic Institution; 12 gifts from the authors, 19 from the publishers, 10 from Mr. John Crane, three standard medical books from Lt. F. G. Hirsch which came from his father's library, and 11 as miscellaneous gifts. There were 20 back sets of serial publications completed: five, purchased by the Marine Biological Laboratory (two with "Carnegie Fund"); three, by the Woods Hole Oceanographic Institution; one secured by exchange with the "Biological Bulletin"; five by exchange with the Woods Hole Oceanographic publications; one as a gift from Lt. F. G. Hirsch; and five secured by duplicate material exchange and by gift. Partially completed sets were 50: purchased by the Marine Biological Laboratory, 23 (14 with "Carnegie Fund); three by the Woods Hole Oceanographic Institution; by exchange of the "Biological Bulletin," none; by exchange of the Woods Hole Oceanographic Institution publications, three; by gift and by exchange of duplicate material, 21.

The reprint additions to the library number 7927: current of 1941, 933; current of 1942, 1471; current of 1943, 381; and of previous dates, 5142. A total of 981 reprints, 296 not duplicates of our holdings, were presented to the library: 396 by Dr. H. E. Crampton; 63 by Dr. H. S. Hopkins; and 522 by Dr. Libbie H. Hyman.

At the end of the year 1943 the library contained 51,945 bound volumes and 130,650 reprints.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of The Marine Biological Laboratory:

Gentlemen:

I beg to submit herewith a report of the fifty-sixth session of the Marine Biological Laboratory for the year 1943.

1. *Research*

During the past year research and instruction have gone on as usual, but the exigencies of war teaching and war research, and the draft have cut deeply into our attendance. There were 108 investigators as compared with an average of 366 in the five years preceding the war. The number of Library readers was somewhat larger than before. In most cases, the length of stay at the Laboratory was shorter than in normal years. The Friday Evening Lectures were continued as usual, but no seminars were held, nor was there a general scientific meeting at the end of the season. Research directly connected with the war was directed by Drs. D. E. S. Brown, Clowes, Heilbrunn, and Jacobs. Other projects were sponsored by the Oceanographic Institution, the Massachusetts General Hospital, and by the Bell Telephone Laboratories. During the Spring of 1944, a number of investigators from the Oceanographic Institution, engaged in war research, have occupied some of our Laboratory rooms.

2. *Instruction*

The Laboratory has been able to continue its courses of instruction with able teaching staffs and with a reasonable number of students. Of a total of 68 students in all of the courses, 11 were men and 57 were women. In peace times the total has been about 125, somewhat less than half being men. The Invertebrate course, under Dr. Buck and an almost entirely new corps of instructors, was successfully given to a class of almost maximum size. Dr. Hamburger and Dr. Costello, with the occasional help of Drs. Barth, Metz, and Rose, carried on the Embryology course with excellent results. Pressure of war work prevented most of the instructors in Physiology from remaining in residence during the entire period of the course, but in spite of this, Dr. Parpart and his associates were able to conduct the course satisfactorily. It was a great disappointment that the course in Algae had to be omitted because of a lack of students. This is the first time since the course first began in 1889 that instruction in Botany has not been given at this Laboratory.

3. *Buildings and Grounds*

With the abatement of danger from enemy attacks in this region, the Navy closed its Base at Woods Hole and gave back to us, on January 1, 1944, our buildings which they had occupied since May 1942. During their tenancy they made a number of alterations, many of which are of permanent value. The Lecture Hall and the Botany Building were given new roofs and shingles and solid shorings to support the floors. In the Homestead new sills and other timbers, and floors, all greatly needed, were installed. The Mess was improved by a new roof and insulated walls, a completely rebuilt ice box and store room, and by the addition of two new insulated store rooms. At the Penzance Garage a new concrete floor was laid where the old one was imperfect. In addition, the water front which had been weakened by the hurricane and only partially repaired, was thoroughly rebuilt. These improvements, which we should have had to make sooner or later, are conservatively valued at $12,000.

On the other hand, the Navy made other alterations which rendered the buildings unsuitable for our use. In the lease, the Navy agreed to restore the property to its original condition. But it was obvious that the necessary repairs and replacements could be made more satisfactorily by our own staff than by an outside contractor. Consequently, after a period of bargaining, it was agreed that the Navy should leave in place some valuable equipment, useful to the Laboratory, in lieu of actual payment for the cost of the repairs. Among the items transferred to us may be mentioned a series of gas cooking ranges equipped with a ventilating hood, and an oven. These take the place of our old coal range which was outworn. Also a water cooler, a refrigerating unit in the icebox, a hot water heating system, extensive plumbing installations in the Homestead, and 12 tons of hard coal in the Apartment House. This equipment is valued at more than $6000, an amount well above the cost of repairs and replacements made by our staff. The Lecture Hall and the Botany Building have not been completely restored since they will not be needed in 1944. The Apartment House, which was returned to use in fairly good condition, has been redecorated for the first time since it was built in 1927. The Penzance Garage is now leased to the Oceanographic Institution for the duration.

Although a number of our buildings are now in better condition than heretofore, some need further attention. Specifically, the walls of the Apartment House should be waterproofed, at least on those sides exposed to storms. Unless this is done soon much of the new decoration will be marred. The Brick Building also should be similarly treated.

4. *Financial*

The Treasurer's report shows that our finances are in some respects in excellent condition. The cash balance is larger than usual, due in part to the large dividend of the General Biological Supply House and increased sales by our own Supply Department, but especially to the rental paid by the Navy. At the same time our expenditures have been low for the same reasons that were noted last year, namely, that we have been unable to purchase new equipment and to pay for current foreign journals. Because of this favorable cash balance we have felt justified in adding a substantial amount to the Reserve Fund, and in making the extensive improvements in the Apartment House.

On the other hand, our income for 1944 will be reduced by reason of the withdrawal of the Navy and the relatively small return from fees of investigators and students. We cannot expect any considerable increase in our regular sources of income. Our present prosperity is therefore transient. During the next few years we must provide funds, in addition to the regular budget, to pay for foreign journals held in store for the duration, for the development of the Apparatus Department, including the services of a full-time mechanic, and especially for the building up of the Retirement Fund which is now far too small. The Executive Committee is considering the last item. The Committee on Additional Funds finds that the present time is not favorable for obtaining money, but it has laid plans for the future.

5. *Losses by Death*

In the death of Dr. Caswell Grave the Laboratory loses one of its most loyal members, whose important services, willingly given during a period of more than 30 years, are gratefully remembered.

Prof. A. D. Morrill, Emeritus Professor of Biology at Hamilton College, active in the early days of the Laboratory, died June 8, 1943.

Pof. John M. McFarlane, Emeritus Professor of Botany at the University of Pennsylvania, Trustee of the Corporation from 1897 to 1902, died September 16, 1943.

6. *Gifts*

The Laboratory is indebted to Mr. John Crane, for a letter written by his father, Charles R. Crane, to Mr. John D. Rockefeller, Jr., on the occasion of the setting up of the Endowment Fund to which both men had generously contributed. In the letter he refers in the following words to the unusual character of the Laboratory when he first become acquainted with it. "These scientists were struggling and accomplishing marvelous things with most meagre equipment, making many sacrifices. It seemed to me that the precious thing to preserve was the spirit of the organization, a spirit everywhere recognized although hard to seize or to imitate. So we have been most careful not in any way to jeopardize this spirit: and processes of organization and management have continued as I found them. It is a valuable expression of a democracy of educated, high minded men."

7. *Election of Trustees*

At the meeting of the Corporation held August 10, 1943, the following Trustees were elected Trustees Emeritus:

B. M. Duggar, The University of Wisconsin
W. E. Garrey, Vanderbilt University Medical School

The new Trustees elected at that meeting are:

Paul S. Galtsoff, U. S. Fish and Wild Life Service
E. W. Sinnott, Professor of Botany, Yale University

8. There are appended as parts of this report:

1. Appreciation of the Services of Professor Frank R. Lillie.
2. Memorials to deceased Trustees.
3. The Staff.
4. Investigators and Students, 1943.
5. A Tabular View of Attendance, 1939–1943.
6. Subscribing and Co-operating Institutions.
7. Evening Lectures.
8. Members of the Corporation.

Respectfully submitted,

CHARLES PACKARD,
Director

1. APPRECIATION OF THE SERVICES OF PROFESSOR FRANK R. LILLIE TO THE MARINE BIOLOGICAL LABORATORY

Read at the Meeting of the Corporation, August 10, 1943

In the history of the Marine Biological Laboratory the names of two men are pre-eminent: Dr. Whitman, who with prophetic insight, envisioned this institution as a national center of research in every department of Biology; and Dr. Lillie, who transformed that vision into reality. Coming to Woods Hole first in 1891 as an investigator receiving instruction, Dr. Lillie, with Dr. Whitman, organized the course in Embryology in 1893. He was appointed Assistant Director in 1900 at a time when the fortunes of the Laboratory were at a low ebb; Director in 1908; and President of the Corporation in 1926, after the successful conclusion of the campaign to obtain new buildings and an endowment. During the period from 1900 to 1942, when he resigned from the Presidency, the Marine Biological Laboratory developed from a struggling organization to its present position as the leading cooperative laboratory of the world.

It is of course true that only by the devoted work of the members of the Corporation, and the active interest of its many friends, could such an end be reached; but it is equally true that without wise guidance this effort would have failed. From the beginning, when Whitman, against every force and discouragement, fought for the principles of co-operation and independence, this Laboratory has pursued its steady course, adapting itself wisely to new conditions as they arose, but always holding to those basic ideals. During his fruitful years as Director, Dr. Lillie frequently stressed these principles. "Our purpose," he wrote, "is essentially ideal, and its pursuit demands our best efforts and our loyalty." And again, "We have laid the principle of co-operation at our foundation, and we have attempted to build it into every one of our activities." In this course he has always quietly led. There has never been any though of division since he has been in charge. Here lies his strength, and here lies the secret of the continued success of the Laboratory.

In accepting his resignation from the Presidency, the Corporation and the Trustees are rejoiced that he will continue his connection with the Laboratory as President Emeritus. We extend to him and to Mrs. Lillie, who has so ably assisted him in the development of the Marine Biological Laboratory, our grateful thanks, and we pledge to him our best efforts to continue the work which he has so long and so wisely guided.

C. E. McClung
E. G. Conklin
Charles Packard

2. MEMORIALS TO DECEASED TRUSTEES

1. Memorial to Dr. Hermon Carey Bumpus

Read by Dr. A. P. Mathews

Hermon Carey Bumpus, Trustee Emeritus of the Marine Biological Laboratory, died June 21, 1943, at the age of eighty-one years. The Laboratory thus loses a member who played an active part in its development for more than forty years. Coming first to Woods Hole in 1889 when a graduate student of Whitman at Clark University, he worked here on his thesis, "The Embryology of the American Lobster." In 1890 he returned to Brown University, his Alma Mater, where he taught Comparative Anatomy for eleven years. It was during this period that he served at this Laboratory as head of the Invertebrate Zoology course; as Assistant Director from 1893 to 1895; and as Clerk of the Corporation from 1897 to 1899. He was a Trustee from 1897 to 1932 when he became Trustee Emeritus.

From 1898 to 1901 he was the Director of the Laboratory of the Fish Commission at Woods Hole, during which time he made a careful survey of the fauna of his region. At this time also he made one of the first studies of variation and its bearing on Natural Selection.

Many of his students from Brown came to us with him, among whom were George M. Gray, A. D. Mead, H. W. Walter, and F. P. Gorham.

Dr. Bumpus was remarkably efficient in the work of organization in all the positions which he held. At this Laboratory he greatly improved the equipment for work and for collecting living material; and due to his efforts the number of students at the Laboratory greatly increased, 85 being registered one year in the Invertebrate Course.

To bring Biology to the people was his chief interest. Accordingly he left Brown in 1901 to become Director of the American Museum of Natural History in New York, where he remained ten years and carried out his long cherished plans for taking the resources of the museum to the school children in New York City, an educational project which has since grown to great proportions throughout the country. Subsequently, while in the National Park Service, he developed many museums in the State and National Parks. In recognition of this work he received the Pugsley Medal for his service to education.

For three years he was Business Manager of the University of Wisconsin, and for five years, President of Tufts College. He was a Trustee also of several charitable institutions.

These are only a few of the many accomplishments of this tireless worker. He was, in the words of his student and life-long friend, Dr. H. E. Walter, "A natural teacher, and enthusiastic scientist, a remarkable executive, and a genial gentleman."

II. MEMORIAL TO DR. G. N. CALKINS

By Dr. L. L. Woodruff

The distinguished incumbent of the first Professorship of Protozoology in America, Gary Nathan Calkins, died at his home in Scarsdale, New York, on January 4, 1943, after a considerable period of ill health which was endured with characteristic cheerfulness and fortitude.

Calkins was born at Valparaiso, Indiana, on January 18, 1869, but spent nearly all of his life on the Eastern seaboard. His scientific training began at the Massachusetts Institute of Technology where, under the influence of Professor William T. Sedgwick, an interest was aroused in biology as a profession. After receiving the B.S. degree in 1890 he served until 1893 as lecturer at the Institute and also as Assistant Biologist to the Massachusetts State Board of Health. Then he transferred to Columbia University to study under Professor Edmund D. Wilson and received the Ph.D. degree in 1898. While a graduate student he was appointed to the teaching staff and thus began the life-long membership in the Department of Zoology at Columbia, which in 1907 culminated as Professor of Protozoology. Calkins was for some years the Executive Officer of the Department, and retired in 1940 as Professor Emeritus in residence. Columbia University conferred on him the honorary degree of Sc.D. in 1929.

Calkins' devotion of his life to the study of the Protozoa was inspired both by an inherent interest in the "little animals," and the well-founded belief that they afford highly favorable material for the approach to many general biological problems. This is best exemplified by his most important treatise, *The Biology of the Protozoa* (1926, 2nd edition 1933), and his long-continued studies on the physiology and cytology of free-living Ciliates, with particular reference to the significance of fertilization and other factors influencing longevity. In this classic work he devised more exact methods of pedigreed culture, involving daily isolation of the animals, that laid the foundations for present-day technique in the field, and he also developed what may be referred to as his philosophy of the Protozoan individual. Both phases stimulated many investigators to enter similar fields.

The extensive series of important studies from Calkins' laboratory is but one of his many contributions to science. A brilliant lecturer and teacher at Columbia and at The Marine Biological Laboratory, his courses revealed a comprehensive grasp of protozoology from both its theoretical and practical aspects that inspired many students; and his versatility was shown by numerous other activities. Thus, for example, he was Consulting Biologist to the New York State Cancer Laboratory at Buffalo from 1902 to 1908, Lecturer before the Lowell Institute in 1907, President of the Association for Cancer Research in 1916, President of the Society for Experimental Biology and Medicine from 1919 to 1921, and Director of the University Union in Paris in 1926 and 1927. He was elected in 1919 to the National Academy of Sciences.

Calkins' association with The Marine Biological Laboratory began just over a half century ago, and for about forty years he was in regular attendance as an investigator. He was a member of the Corporation for 39 years and its Clerk for 17 years, member of the Board of Trustees for 30 years and its Secretary for 12 years, member of the Research Staff for 31 years, and head of the Protozoology course, which he founded, for 22 years.

Zoology in general and Columbia University and The Marine Biological Laboratory in particular owe to Calkins more than can be readily expressed for his scientific contributions, teaching, and administrative service. All this, as well as his personal charm, unfailing enthusiasm, and hearty good fellowship, was attested by his former students and associates who presented to him after retirement a volume of nearly two hundred letters of esteem and appreciation inscribed:

Gary Nathan Calkins
Philosopher in Little Things
and Friend.

3. THE STAFF, 1943

CHARLES PACKARD, Director, Marine Biological Laboratory, Woods Hole, Massachusetts.

SENIOR STAFF OF INVESTIGATION

E. G. CONKLIN, Professor of Zoology, Emeritus, Princeton University.
CASWELL GRAVE, Professor of Zoology, Emeritus, Washington University.
FRANK R. LILLIE, Professor of Embryology, Emeritus, The University of Chicago.
RALPH S. LILLIE, Professor of General Physiology, Emeritus, The University of Chicago.
C. E. MCCLUNG, Professor of Zoology, Emeritus, University of Pennsylvania.
S. O. MAST, Professor of Zoology, Emeritus, Johns Hopkins University.
A. P. MATHEWS, Professor of Biochemistry, Emeritus, University of Cincinnati.
T. H. MORGAN, Director of the Biological Laboratory, California Institute of Technology.
G. H. PARKER, Professor of Zoology, Emeritus, Harvard University.

ZOOLOGY

I. CONSULTANTS

T. H. BISSONNETTE, Professor of Biology, Trinity College.
L. L. WOODRUFF, Professor of Protozoology, Yale University.

II. INSTRUCTORS

J. B. BUCK, Assistant Professor of Zoology, University of Rochester, in charge of course.
W. D. BURBANCK, Associate Professor of Biology, Drury College.
M. D. BURKENROAD, Assistant Curator, Bingham Oceanographic Foundation, Yale University.
C. G. GOODCHILD, Associate Professor of Biology, Southwest Missouri State Teachers College.
RONALD GRANT, Lecturer in Physiology, McGill University.
JOHN H. LOCHHEAD, Instructor in Zoology, University of Vermont.
MADELENE E. PIERCE, Assistant Professor of Zoology, Vassar College.
MARY D. ROGICK, Professor of Biology, College of New Rochelle.

III. LABORATORY ASSISTANT

MARGARET L. KEISTER, Instructor in Biology, Wheaton College.

EMBRYOLOGY

I. CONSULTANTS

L. G. BARTH, Assistant Professor of Zoology, Columbia University.
H. B. GOODRICH, Professor of Biology, Wesleyan University.

II. INSTRUCTORS

VIKTOR HAMBURGER, Professor of Zoology, Washington University, in charge of course.
DONALD P. COSTELLO, Assistant Professor of Zoology, University of North Carolina.
CHARLES B. METZ, Instructor, Wesleyan University.

PHYSIOLOGY

I. CONSULTANTS

WILLIAM R. AMBERSON, Professor of Physiology, University of Maryland, School of Medicine.

GENERAL MAINTENANCE

T. E. Larkin, Superintendent

W. C. Hemenway | T. E. Tawell
R. W. Kahler | J. Wynne
L. J. Pierce

THE GEORGE M. GRAY MUSEUM

George M. Gray, Curator Emeritus

4. INVESTIGATORS AND STUDENTS

Independent Investigators, 1943

Addison, William H. F., Professor of Normal Histology and Embryology, University of P sylvania, School of Medicine.

Ball, Eric G., Associate Professor of Biological Chemistry, Harvard Medical School.

Barth, L. G., Assistant Professor of Zoology, Columbia University.

Berger, Charles A., Director, Biological Laboratory, Fordham University.

Brooks, Matilda M., Research Associate in Biology, University of California

Brown, Dugald E. S., Professor of Physiology, New York University, College of Dentistr

Buck, John B., Assistant Professor of Zoology, University of Rochester.

Budington, Robert A., Professor of Zoology, Emeritus, Oberlin College.

Buggs, Charles W., Professor of Biology and Head of Division of the Sciences, Dillard versity.

Burbanck, William D., Associate Professor and Chairman of Department of Biology, D College.

Burkenroad, Martin D., Assistant Curator, Bingham Oceanographic Foundation, Yale versity.

Chambers, Robert, Research Professor of Biology, Washington Square College, New University.

Clark, Eleanor L., Voluntary Investigator, University of Pennsylvania, School of Medicin

Clark, E. R., Professor and Head of Department of Anatomy, University of Pennsylva School of Medicine.

Clement, Anthony C., Associate Professor of Biology, College of Charleston.

Clowes, G. H. A., Director of Research, Eli Lilly and Company.

Conklin, Edwin G., Professor of Biology, Emeritus, Princeton University.

Costello, Donald P., Associate Professor of Zoology, University of North Carolina.

Duggar, Benjamin M., Professor of Plant Physiology, University of Wisconsin.

Evans, Titus C., Assistant Professor of Radiology, College of Physicians and Surgeons, lumbia University.

Failla, G., Professor of Radiology, College of Physicians and Surgeons, Columbia Univer

Fisher, Kenneth C., Assistant Professor of Physiological Zoology, University of Toront

Gabriel, Mordecai L., Department of Zoology, Columbia University.

Galtsoff, Paul S., Senior Biologist, U. S. Fish and Wildlife Service.

Garrey, W. E., Professor of Physiology, Vanderbilt University, School of Medicine.

Glaser, Otto C., Professor of Biology, Amherst College.

Goodchild, Chauncey G., Associate Professor of Biology, State Teachers College, Springf Missouri.

Grand, C. G., Research Associate, Washington Square College, New York University.

Granick, Sam, Assistant, Rockefeller Institute.

Grant, Ronald, Lecturer in Zoology and Physiology, McGill University.

Grave, Caswell, Professor of Zoology, Emeritus, Washington University.

Hamburger, Viktor, Professor of Zoology, Washington University.
Harnly, Morris H., Associate Professor of Biology, Washington Square College, New York University.
Harris, Daniel L., Research Associate, University of Pennsylvania.
Hartman, Frank A., Professor and Chairman of Department of Physiology, Ohio State University.
Harvey, Ethel B., Independent Investigator in Biology, Princeton University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Heilbrunn, L. V., Associate Professor of Zoology, University of Pennsylvania.
Hiatt, Edwin P., Assistant Professor, New York University, College of Dentistry.
Hopkins, Hoyt S., Associate Professor of Physiology, New York University, College of Dentistry.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania, School of Medicine.
Kempton, Rudolf T., Professor of Zoology, Vassar College.
Krahl, M. E., Chemist, Eli Lilly and Company.
Lavin, George, Rockefeller Institute for Medical Research.
Lee, Richard E., Research Associate, Washington Square College, New York University.
Lillie, Frank R., Professor of Zoology, Emeritus, The University of Chicago.
Lillie, Ralph S., Professor of Physiology, Emeritus, The University of Chicago.
Little, E. P., Instructor in Science, Phillips Exeter Academy.
Lochhead, John H., Instructor in Zoology, University of Vermont.
McClung, C. E., Professor of Zoology, Emeritus, University of Pennsylvania.
McElroy, William D., Research Associate, Princeton University.
Marsland, Douglas A., Associate Professor of Biology, Washington Square College, New York University.
Mast, S. O., Professor of Zoology, Emeritus, Johns Hopkins University.
Mathews, Albert P., Professor of Biochemistry, Emeritus, University of Cincinnati.
Meglitsch, Paul, Head of Department of Zoology, Herzl Junior College.
Memhard, Allen R., Crescent Road, Riverside, Connecticut.
Metz, Charles B., Instructor in Biology, Wesleyan University.
Michaelis, Leonor, Rockefeller Institute for Medical Research.
Morgan, Lillian V., California Institute of Technology.
Morgan, T. H., Professor of Biology, California Institute of Technology.
Nelson, Leonard, Medical Student, University of Pennsylvania.
Osterhout, W. J. V., Member Emeritus, Rockefeller Institute for Medical Research.
Packard, Charles, Director, Marine Biological Laboratory.
Parker, George H., Professor of Zoology, Emeritus, Harvard University.
Parmenter, Charles L., Professor of Zoology, University of Pennsylvania.
Parpart, A. R., Associate Professor of Biology, Princeton University.
Pierce, Madelene E., Assistant Professor of Zoology, Vassar College.
Pollister, Arthur W., Associate Professor of Zoology, Columbia University.
Reinhard, Edward G., Head, Department of Biology, Catholic University of America.
Rogick, Mary D., Professor of Biology, College of New Rochelle.
Rose, S. Meryl, Instructor in Zoology, Smith College.
Russell, Alice M., Instructor in Zoology, Fordham University.
Schaeffer, A. A., Professor of Biology, Temple University.
Schallek, William B., Fellow, Woods Hole Oceanographic Institution.
Scott, Sister Florence M., Professor of Biology, Seton Hill College.
Sichel, Elsa K., Head of Science Department, Vermont State Normal School.
Sichel, F. J. M., Assistant Professor of Physiology, University of Vermont College of Medicine.
Slifer, Eleanor H., Assistant Professor of Zoology, State University of Iowa.
Sosa, Julio M., Professor of Histology and Embryology, College of Medicine, Montevideo, Uruguay.
Steinbach, H. Burr, Associate Professor of Zoology, Washington University.
Stokey, Alma G., Professor of Botany, Emeritus, Mount Holyoke College.

Stunkard, Horace W., Professor of Biology, New York University.
Taylor, William R., Professor of Botany, University of Michigan.
Wenrich, D. H., Professor of Zoology, University of Pennsylvania.
Whiting, Anna R., Guest Investigator, University of Pennsylvania.
Whiting, P. W., Associate Professor of Zoology, University of Pennsylvania.
Wiercinski, Floyd J., Instructor in Biology, Catholic University of America.
Wrinch, Dorothy, Research Associate in Physics, Smith College.
Zweifach, Benjamin W., Research Associate in Biology, Washington Square College, New York University.

Research Assistants, 1943

Abramsky, Tessie, Technician, Rockefeller Institute for Medical Research.
Behan, Anne, Research Assistant, Columbia University.
Gidge, Natalie, Smith College.
Heidenthal, Gertrude, Research Assistant, University of Pennsylvania.
Honegger, Carol, Research Assistant, Temple University.
Hutchinson, Dorothea, Cazenovia Junior College.
John, Hedda Maria, Research Assistant in Neurology, Columbia University.
Kawata, Nobuyuki, Research Assistant, Washington University.
Krugelis, Edith, Research Assistant, Columbia University.
Lawler, Helen Claire, Research Assistant, New York University.
Lowenhaupt, Marian, Graduate Student, Washington University.
Miner, Karyl R., New York University.
Odlaug, T. O., Research Biologist, Fish and Wildlife Service.
Quinn, Gertrude Patricia, Research Assistant, New York University, College of Dentistry.
Stern, Joseph R., University of Toronto.
Woodward, Arthur A., Jr., Research Assistant, University of Pennsylvania.
Yarnall, Margaret, Student, University of Pennsylvania.

Library Readers, 1943

Amberson, William R., Professor of Physiology, University of Maryland Medical School.
Anderson, Thomas F., Associate, Johnson Foundation, University of Pennsylvania.
Beck, Lyle V., Instructor in Physiology, Hahnemann Medical College.
Boche, Robert D., Instructor in Zoology, University of Pennsylvania.
Botsford, E. Frances, Associate Professor of Zoology, Connecticut College.
Brodie, Bernard B., Research Associate in Biochemistry, New York University.
Brownell, Katharine A., Research Associate in Physiology, Ohio State University.
Cahen, Raymond L., Research Assistant in Pharmacology, Yale University Medical School.
Cahn, T., New York University.
Cahnmann, Hans J., Research Associate, Mt. Sinai Hospital.
Cassidy, Harold G., Instructor in Organic Chemistry, Yale University.
Chidsey, Jane L., Assistant Professor of Zoology, Wheaton College.
Croasdale, Hannah T., Technical Assistant, Dartmouth College.
Franklin, Roger G., Professor of Biology, St. Joseph's Seminary.
Furth, Jacob, Associate Professor of Pathology, Cornell University Medical College.
Gates, R. Ruggles, Professor of Botany, Emeritus, University of London.
Gurewich, Vladimir, Clinical Assistant and Attending Physician, Cornell Division of the Bellevue Hospital.
Haywood, Charlotte, Professor of Physiology, Mount Holyoke College.
Hibbard, Hope, Professor of Zoology, Oberlin College.
Krasnow, Frances, Head, Department of Biochemistry, Guggenheim Dental Clinic.
Lilly, John C., Fellow in Biophysics, University of Pennsylvania.
Lowenstein, Otto, Clinical Professor of Neurology, New York University.
Loewi, Otto, Research Professor, New York University, College of Medicine.
Marshall, Helen A., Massachusetts State College.
Mavor, James W., Professor of Biology, Union College.

Menkin, Valy, Assistant Professor of Pathology, Harvard Medical School.
Meyerhof, Otto, Research Professor of Biochemistry, University of Pennsylvania.
Moldaver, Joseph, Research Assistant in Neurology, Columbia University.
Nachmansohn, David, Research Associate in Neurology, Columbia University.
Schmitt, Francis O., Head, Department of Biology and Biological Engineering, Massachusetts Institute of Technology.
Silow, Ronald A., Geneticist, Cotton Research Station, Trinidad.
Stahmann, Mark A., Research Assistant in Chemistry, Rockefeller Institute for Medical Research.
Stern, Kurt G., Chief Chemist, Overly Biochemical Research Foundation.
Wolf, Opal M., Associate Professor, Goucher College.
Woodward, Alvalyn E., Assistant Professor of Zoology, University of Michigan.

Beginning Investigators, 1943

Burden, Rhoda L., Fellow in Biology, Tufts College.
Grell, Sister Mary, Graduate Student, Fordham University.
Hinchey, M. Catherine, Instructor in Biology, Temple University.
Hopkins, Alice, Student, University of Rochester.
Jacobs, Athleen R., Teaching Fellow in Biology, Radcliffe College.
Jaeger, Lucena, Graduate Student, Columbia University.
Jenkins, Janet, Student, Wheaton College.
Keister, Margaret L., Instructor in Zoology, Wheaton College.
Lawniezak, Sister Mary J., Student, Fordham University.
LeFevre, Paul G., Research Assistant, University of Pennsylvania.
Lehman, Gene, Graduate Teaching Fellow, University of North Carolina.
Littrell, Jae L., Teaching Assistant, University of Illinois.
Marks, Mildred H., Research Assistant, University of Pennsylvania.
Mortensen, Edith, Assistant Professor of Zoology, George Washington University.
Philbrick, Madeline G., Student, Russell Sage College.
Price, Winston H., Student, University of Pennsylvania.
Satake, Jack, Graduate Student, Washington University.
Wilson, Walter L., Graduate Student, University of Pennsylvania.
Witkus, Eleanor R., Research Associate, Fordham University.

Students, 1943

EMBRYOLOGY

Banks, Mary E., Undergraduate Assistant, Washington University.
Cole, Elsie L., Graduate Teaching Assistant, University of Wisconsin.
Hopkins, Alice, Student, University of Rochester.
Jenkins, Janet R., Undergraduate Assistant, Wheaton College.
Lehman, Gene, Teaching Fellow, University of North Carolina.
Loose, Marian, Graduate Assistant in Biology, Goucher College.
Lowenhaupt, Marian, Student, Washington University.
Mekeel, Amy G., Cornell University.
Satake, Jack N., Graduate Student, Washington University.
Travis, Dorothy F., George Washington University.
Wahlert, Mary R., Washington University.
Wilde, Betty L., Laboratory Technician, Rockefeller Institute for Medical Research.
Wolf, Thema M., Washington University.

PHYSIOLOGY

Burden, Rhoda L., Graduate Assistant, Tufts College.
France, Horace O., Research Fellow in Biology, Colorado University.
Philbrick, Madeline G., Russell Sage College.

Reilly, Sister Sara L., Seton Hill College.
Schneyer, Leon H., 101 Magnolia Avenue, Mount Vernon, New York.
Siegel, Blossom, Student, Brooklyn College.
Tashiro, Mitsuko, Graduate Assistant, Vassar College.
Wood, Marcia, Russell Sage College.

ZOOLOGY

Andrus, Mary, Student, Wellesley College.
Baca, Antoinette, Duke University.
Baker, Laura J., Graduate Student, University of Rochester.
Bourquin, Phyllis J., Student, Oberlin College.
Brooks, Barbara, Radcliffe College.
Brooks, Edith H., Student, Swarthmore College.
Chapman, Mary P., Student, Vassar College.
Chew, Robert M., Student, Washington and Jefferson College.
Crane, Lois H., Vassar College.
Dehner, Eugene W., Instructor in Biology, St. Benedict's College.
Ely, Charles A., Graduate Assistant, Washington and Jefferson College.
Getz, Charlotte E., Student, University of Chicago.
Graham, K. Merle, Assistant in Zoology, University of Illinois.
Grell, Sister Mary, Student, Fordham University.
Hamilton, Helen R., Science Teacher, Emma Willard School.
Harnischfeger, Eleanor, Student, Washington College.
Hegemann, Idolene, Student, Bennington College.
Hignutt, Charlotte R., Student, Washington College.
Holloway, Rita H., Student, Oberlin College.
Holmes, Thelma M., Seton Hill College.
Honegger, Carol M., Student, Temple University.
Jan Tausch, Anne M., Student, New Jersey College for Women.
Jensen, Dorothy, Mount Holyoke College.
Katsafanas, Georgia, Student, Seton Hill College.
Kelley, Ellen M., Student, New Jersey College for Women.
Justitia, Sister Mary, Student, Fordham University.
Lieb, Margaret, Student, Smith College.
Marsh, Mary G., Vassar College.
Murray, Helen E., Student, Emmanuel College.
Paisley, Anne, Student, DePauw University.
Pope, Edith, Student, Smith College.
Pope, Louise S., Instructor in Biology, Whitman College.
Pope, Philip H., Professor of Biology, Whitman College.
Ransom, Gladys Virginia, Biology Laboratory Assistant, Wilson College.
Reich, Eva, Student, Barnard College.
Richards, Philip W., Groton School.
Rigaumont, Jean A., Student, Pennsylvania College for Women.
Rothrock, Suzanne, DePauw University.
Rubright, Eleanor, Student, Ohio Wesleyan University.
Somers, Elizabeth F., Goucher College.
Taylor, Ruth M., Student, Oberlin College.
Uphoff, Delta E., Student, Russell Sage College.
Weber, Kathleen K., Student, University of Richmond.
Williams, Patricia A., Student, Seton Hill College.
Witkus, Eleanor R., Research Associate, Fordham University.
Yamamato, Kekahuna H., DePauw University.
Zarudnaya, Katerina I., Graduate Assistant, Johns Hopkins University.

5. TABULAR VIEW OF ATTENDANCE

	1939	*1940*	*1941*	*1942*	*1943*
Investigators—Total	352	386	337	201	160
Independent	213	253	197	132	89
Under instruction	60	62	59	16	19
Research assistants	79	71	50	25	17
Library readers	—	—	31	28	35
Students—Total	133	128	131	74	68
Zoology	55	55	55	36	47
Protozoology (not given after 1940)	12	7	—	—	—
Embryology	36	34	37	24	13
Physiology	21	22	24	6	8
Botany	9	10	15	8	—
Total Attendance	485	514	468	275	228
Less persons registered as both students and investigators	14	7	7	2	6
	471	507	461	273	222
Institutions Represented—Total	162	148	144	126	116
By investigators	132	112	102	83	70
By students	72	79	72	43	41
Schools and Academies Represented					
By investigators	2	1	5	2	2
By students	2	2	2	–	1
Foreign Institutions Represented					
By investigators	8	2	3	–	2
By students	1	1	1	–	–

6. SUBSCRIBING AND COOPERATING INSTITUTIONS

1943

Amherst College
Atlanta University
Bell Telephone Laboratories, Inc.
Biological Institute, Philadelphia, Pennsylvania
Bowdoin College
Brooklyn College
Bryn Mawr College
Catholic University of America
College of Physicians and Surgeons
Columbia University
Commonwealth Fund
Cornell University
Cornell University Medical College
Duke University
Fish and Wild Life Service, U. S. Dept. of the Interior
Fordham University
Goucher College
Harvard University
Harvard University Medical School
Industrial and Engineering Chemistry, of the American Chemical Society
Johns Hopkins University
Eli Lilly & Co.
Massachusetts General Hospital
Mount Holyoke College
New York University
New York University College of Medicine
New York University Washington Square College
Oberlin College
Ohio State University
Pennsylvania College for Women
Princeton University
Radcliffe College
Rockefeller Institute for Medical Research
Rutgers University
Seton Hill College
Smith College
State University of Iowa
Tufts College
University of Chicago
University of Cincinnati
University of Illinois
University of Maryland Medical School
University of Pennsylvania
University of Pennsylvania School of Medicine

University of Pennsylvania Johnson Foundation
University of Rochester
Vassar College
Villanova College
Washington University
Wellesley College
Wheaton College
Woods Hole Oceanographic Institution
Yale University

7. EVENING LECTURES, 1943

Friday, July 9
Dr. W. R. Taylor .... "Utilization of Marine Plants."
Friday, July 16
Dr. D. P. Costello .... "Experiments on the Localization of Developmental Factors in the Egg of Nereis."
Friday, July 23
Dr. Paul S. Galtsoff .... "Physiology of Sex and Sex Change in the Genus Ostrea."
Friday, July 30
Dr. Rudolf T. Kempton .... "Renal Secretion."
Friday, August 6
Dr. L. V. Heilbrunn .... "The Calcium-Release Theory of Stimulation."
Friday, August 13
Dr. Kurt G. Stern .... "Studies on Iron Proteins."
Friday, August 20
Dr. B. M. Duggar .... "Studies in the Irradiation of Certain Micro-organisms."
Friday, August 27
Mr. A. H. Woodcock .... "Wind-induced Motion of the Physalia."

8. MEMBERS OF THE CORPORATION, 1943

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Maryland.
Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.
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Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
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Evans, Mrs. Glendower, 12 Otis Place, Boston, Massachusetts.
Foot, Miss Katherine, Care of Morgan Harjes Cie, Paris, France.
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King, Mr. Chas. A.
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Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pa.
Morgan, Mrs. T. H., Pasadena, California.
Morgan, Prof. T. H., Director of Biological Laboratory, California Institute of Technology, Pasadena, California.
Noyes, Miss Eva J.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
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Trelease, Prof. William, University of Illinois, Urbana, Illinois.
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Wallace, Louise B., 359 Lytton Avenue, Palo Alto, California.

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Bigelow, Prof. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

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Harvey, Dr. E. Newton, Guyot Hall, Princeton University, Princeton, New Jersey.
Harvey, Dr. Ethel Browne, 48 Cleveland Lane, Princeton, New Jersey.
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Hecht, Dr. Selig, Columbia University, New York City, New York.
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Henshaw, Dr. Paul S., National Cancer Institute, Bethesda, Maryland.
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Kidder, Dr. George W., Brown University, Providence, Rhode Island.

Kille, Dr. Frank R., Swarthmore College, Swarthmore, Pennsylvania.

Kindred, Dr. J. E., University of Virginia, Charlottesville, Virginia.

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King, Dr. Robert L., State University of Iowa, Iowa City, Iowa.

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EMBRYONIC GROWTH IN THE VIVIPAROUS POECILIID, HETERANDRIA FORMOSA [1]

NEVIN S. SCRIMSHAW

(From the Biological Laboratories, Harvard University, Cambridge)

In *Heterandria formosa* the fertilized eggs are minute and the embryos secure their nourishment for development through a placental type of association with the mother. This paper presents an analysis of embryonic growth in this viviparous species and a comparison of this growth with that in oviparous fishes. In the discussion an attempt is made to evaluate the factors restricting growth in Heterandria. A similar study of embryonic growth in a number of ovoviviparous fishes is to follow.

Bailey (1933) and Turner (1937) have directed attention to the development of embryos in poeciliid fishes in relation to the mother. Turner (1940a, b, c, d) surveyed the various types of association between mother and embryo for the four viviparous Cyprinodont families (Poeciliidae, Anablepidae, Goodeidae, and Jenynsiidae).

For the oviparous fishes, the work of Gray (1926, 1928) on the development of the trout *Salmo fario* stands almost alone. However, Kronfeld and Scheminzki (1926) have also contributed data on the trout. This work on a fish species totally dependent upon yolk for its embryonic nourishment provides the basis for the comparison and interpretation of many of the observations described below.

MATERIAL

Heterandria formosa is remarkable in showing a high degree of superfetation. As many as eight broods of developing embryos occur within the ovary of a single female. Active sperm are retained in the ovary for many months following a single copulation. Thus it is impossible to determine the exact time of fertilization or the time it has taken any particular brood to reach the stage in which it is found.

Its embryonic development resembles in its general aspects that of Fundulus and other Cyprinodont fishes. In Heterandria, as in all poeciliid fishes, the embryos are retained until parturition within the follicles of the single median ovary. Following fertilization the ovum and the follicle become separated by the perivitelline space and fluid. The follicular membrane is henceforth generally referred to as the ovisac. Its diameter increases with the extension of the fluid filled spaces associated with the embryo and with the growth of the embryo itself.

Sections of the immature ova show little yolk, but numerous small oil globules are already present (Fig. 1). The latter increase in volume as the eggs grow, and their number is reduced by the coalescence of the small vacuoles (Fig. 2). The large oil globules fuse at the time of fertilization to form a single large spherical oil mass which occupies from 50 to 75 per cent of the volume of the ripe ovum (Fig. 3). When the blastodisc appears, it bears the same relation to the oil globule in Heterandria as it does to the yolk mass in the more typical teleost egg. Thus a central periblast is observed beneath the blastodisc and a thin syncytial periblast eventually surrounds the oil globule in precisely the same manner as it surrounds the yolk in Fundulus.

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[1] The author expresses appreciation to Dr. Leigh Hoadley for suggestions and encouragement in this work.

The chemical composition of the oil globule has not been determined, but it stains deeply with the fat dyes Sharlach R and Sudan III. The great reduction in the amount of the other yolk components present would suggest that the food of the embryo during the first part of its development might be derived from the oil. Figure 4 shows the relation between the diameter of the ovisac and the volume of the oil globule. It indicates that the volume is decreased by less than 30 per cent up to the time that the pseudo-placenta is fully formed.

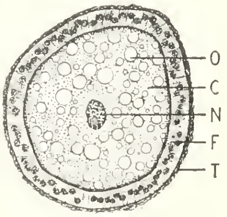

FIGURE 1

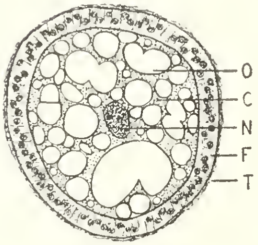

FIGURE 2

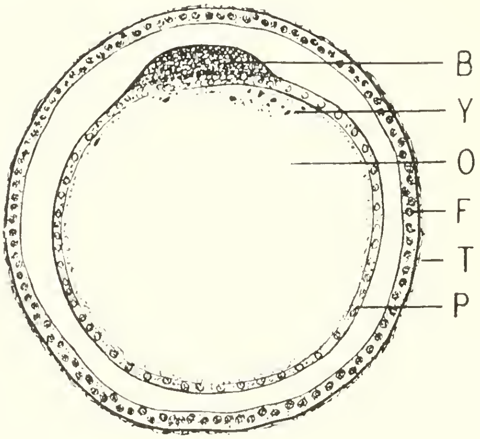

FIGURE 3

FIGURE 1. Early ovum of *Heterandria formosa.* O, oil; C, cytoplasm; N, nucleus; F, follicular epithelium; T, theca.

FIGURE 2. Late ovum of *Heterandria formosa.*

FIGURE 3. Early blastodisc of *Heterandria formosa.* B, blastodisc; Y, yolky periblast; Ch, chorion.

Fraser and Renton (1940) and Turner (1940a) have described the fetal adaptations associated with viviparity in Heterandria. These are unique and may be summarized as follows: The antero-ventral somatopleure of the embryo becomes enormously extended and encloses the head completely. This produces a large extraembryonic pericardial cavity extending into the head fold. The postero-ventral somatopleure is also somewhat expanded and contains the liver, gut and the much extended urinary bladder. An extensive capillary network develops on this expanded portion of the somatopleure so that its whole surface opposing the follicular epithelium is highly vascularized. Since the latter has likewise developed an extensive vascular system, an association between embryonic and maternal circulation comparable to a non-deciduate mammalian placenta is formed.

In relatively advanced embryos the pericardial hood ruptures over the forepart of the head, leaving a strap of somatopleure over it. This strap gradually decreases in size and eventually disappears. As a result the area of the vascular association between embryo and ovisac has been considerably reduced by the time of parturition.

Special types of cells in connection with the maternal and embryonic components of the pseudo-placenta also appear. Turner (1940a) has stated that the outer layer of the extra-embryonic pericardial cavity, the ectoderm, is equipped with "conical cells, which are granular at the base and vacuolated apically . . . and sometimes gathered into low mounds." Their role has not been determined but it is assumed that they aid in some way the nourishment of the embryo through the pseudo-placenta.

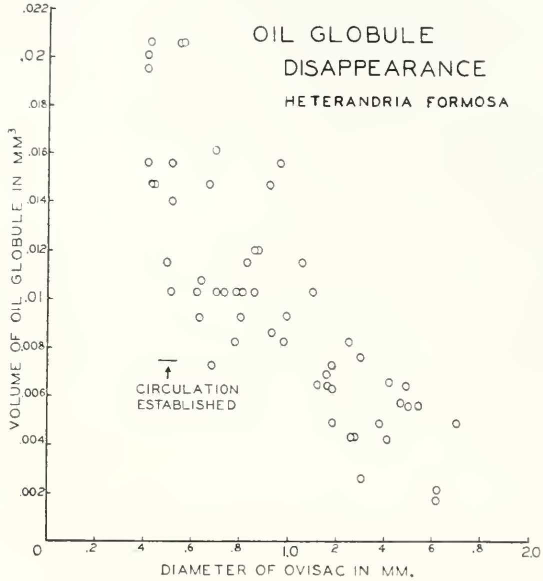

FIGURE 4. The volume in cubic millimeters of the oil globule in fertilized eggs and embryos of Heterandria is plotted as a function of the diameter of the ovisac in millimeters. Each point represents a single pair of readings.

Another cell specialization has not been described previously but seems to become important about the time that the head breaks through the enveloping pericardial sac. It probably represents a supplementary nutritive mechanism which helps to compensate for the reduction in association surface. Sometime during the last quarter of development, the wall of the ovisac, which has appeared as a squamous epithelium since fertilization, hypertrophies. In a restricted region of the ovisac prominent secretory cells are developed. These seem to form an approximately one-third of the surface of the ovisac and parallel more or less closely the embryonic vascular surface. In Figure 5 these cells are seen opposite the blood vessels in the wall of the pericardium, but they may also occur opposite nonvascularized body epithelium. Histologically they appear to be active secretory cells

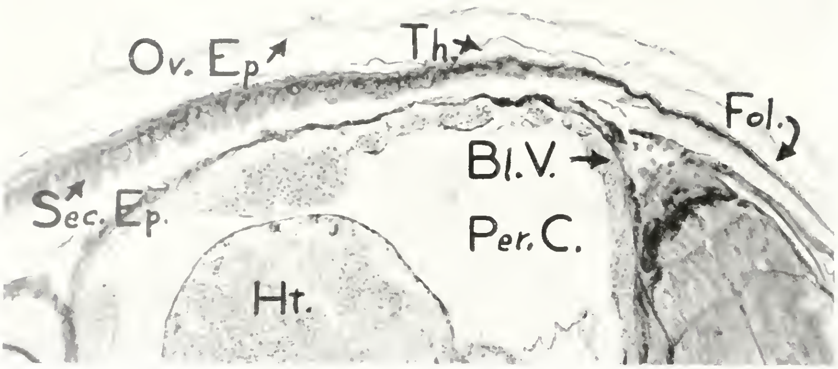

FIGURE 5. Photomicrograph of a section through the ovisac and pericardium of a later embryo of *Heterandria formosa* showing secretory cells of the ovisac epithelium. Ov. Ep., ovarian epithelium; Th., theca; Fol., follicular epithelium; Sec. Ep., secretory portion of follicular epithelium; Bl. V., blood vessel; Per. Cav., pericardial cavity; Ht., heart.

containing many secretion granules. Coagulum which may be from this source appears in sections through the cavity of the ovisac and also in the gut.

METHODS

Both wet and dry weights were determined for members of each brood of embryos taken from 16 females with varied previous histories. One hundred and fifty-one embryos were weighed either individually or in groups belonging to the same brood. The embryos were dissected from the ovary with the ovisac intact. The diameter[2] of the ovisac was measured with an ocular micrometer. The embryos were allowed to touch fine absorbent paper to remove surplus fluid and immediately placed on flamed platinum squares of known weight. These were weighed at once in a single rapid trial to determine the wet weight of the embryo in the intact ovisac. They were then dried over calcium chloride to constant weight.

All weighings were made with a Sartorius balance. The initial weights of the platinum squares and the final weights of the squares plus dried embryos were each determined by 10 to 20 readings (with zero correction before and after each reading). The standard deviation[3] for a given series of readings was rarely over .05 mg. In several trial cases two weeks in the dessicator intervened between the first and second group of ten readings. The standard deviation was still below .05 mg.

The smaller embryos were weighed in groups. The average wet weight of 30 of the earliest embryos was 0.026 mg. After a consideration of all sources of error, this value was estimated to be within at least 0.008 mg. of the true wet weight at the time of fertilization. The dry weight value of 0.017 mgs. was similarly estimated to be within at least 0.002 mgs. of the correct value for this stage. In all of the weight measurements except those on the very early stages the experimental error was small compared to the normal variation of the material.

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[2] The ovisac is spherical at first but becomes increasingly ellipsoidal as development progresses. In the late stages the average of several measurements made on the same ovisac proved to be a reasonably consistent measure of size and was recorded as if it were a true diameter.

[3] $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$.

Ash determinations are impractical for early stages because of the extremely small weights involved. Later stages were ashed in a small muffle furnace and an average ash content of 2 to 3 per cent of the dry weight found.

Weight Changes of the Developing Embryo

Since the age of the embryos was not known, the diameter of the ovisac was selected as a convenient indication of the stage of development, and the weight determinations were correlated with this dimension. When this was done the great increase in weight of the embryo in the intact ovisac was apparent (Fig. 6). The relationship between dry weight and ovisac diameter is repeated on a larger scale for later comparisons (Fig. 7).

Before any appreciable gain in dry weight can be detected the wet weight can be seen to have increased markedly. In fact, the water content of the whole egg plus ovisac increases from 34.6 per cent to 86.8 per cent in this period. There is considerable experimental error in the first figure because of the small weights involved, but a value of 35 per cent is consistent with the large amount of oil present.

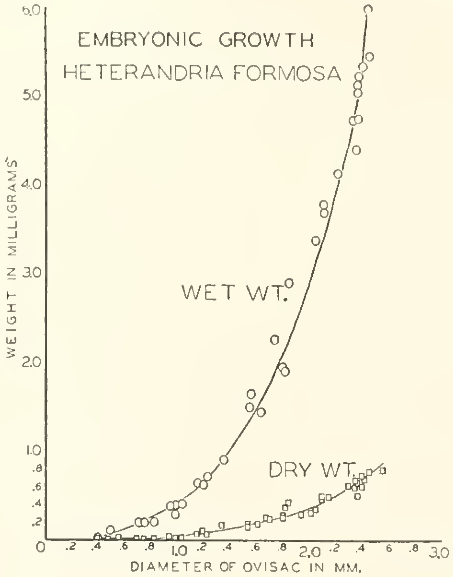

Figure 6. The curves show the increase in both wet and dry weights of developing embryos of *Heterandria formosa* plotted as a function of the diameter of the ovisac, a convenient measure of development.

After the dry weight has begun to increase, the relation between wet and dry weight stays remarkably constant. When the period was divided into two subperiods, the first including embryos with ovisac diameters between 1.00 and 2.00 and the second including embryos with ovisac diameters greater than 2.00, the differences in per cent dry weight were not significant. During the first sub-period the dry weight average 12.84 per cent of the wet weight (σ 1.8%) and during the second it was 13.49 per cent (σ 1.9%). The average was 13.2 per cent.

After parturition the embryo breaks out of the fluid-filled ovisac and the extra liquid in the expanded pericardial cavity disappears. Accordingly the percentage dry weight increases abruptly to a stable value for the young fish of 20.6 per cent (σ 1.7%). This information can be diagrammed to show the changes in composition from the egg at the time of fertilization to the larva at the time of parturition (Fig. 8).

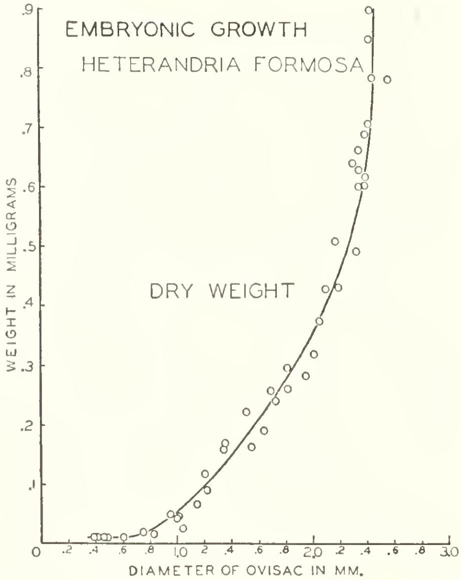

FIGURE 7. The curve shows the increase in the dry weight of embryos of *Heterandria formosa* plotted as a function of the diameter of the ovisac. This represents the same weight values shown in the dry weight curve of Figure 6, but the units of the weight ordinate are much expanded.

These changes can also be represented in the form of a generalized equation which shows the relation of .1 mg. of ovum to the final weight of the larva.

0.1 mg.	+	2.6 mg.	+	15.8 mg.	=	18.5 mg.
(wet weight of egg)		(dry weight from mother)		(wet weight from mother		(wet weight of young fish)

This equation expresses the overall changes. A similar expression has been developed for the trout by Gray (1926). His formula holds from the time the embryo is 50 per cent developed to the time it is 80 per cent formed during which time it has increased about 400 per cent in weight. During this period the trout embryo converts 1.0 grams of yolk (wet weight) plus 0.7 grams of water into 1.56 grams of fish.

Since the yolk makes up almost the entire egg, it can be estimated that one gram of trout egg makes only slightly more than 1.5 grams of trout embryo. In comparison one gram of Heterandria egg would result in 185 grams of embryo. This strikingly illustrates the importance of the maternal contribution of nourishment in this species.

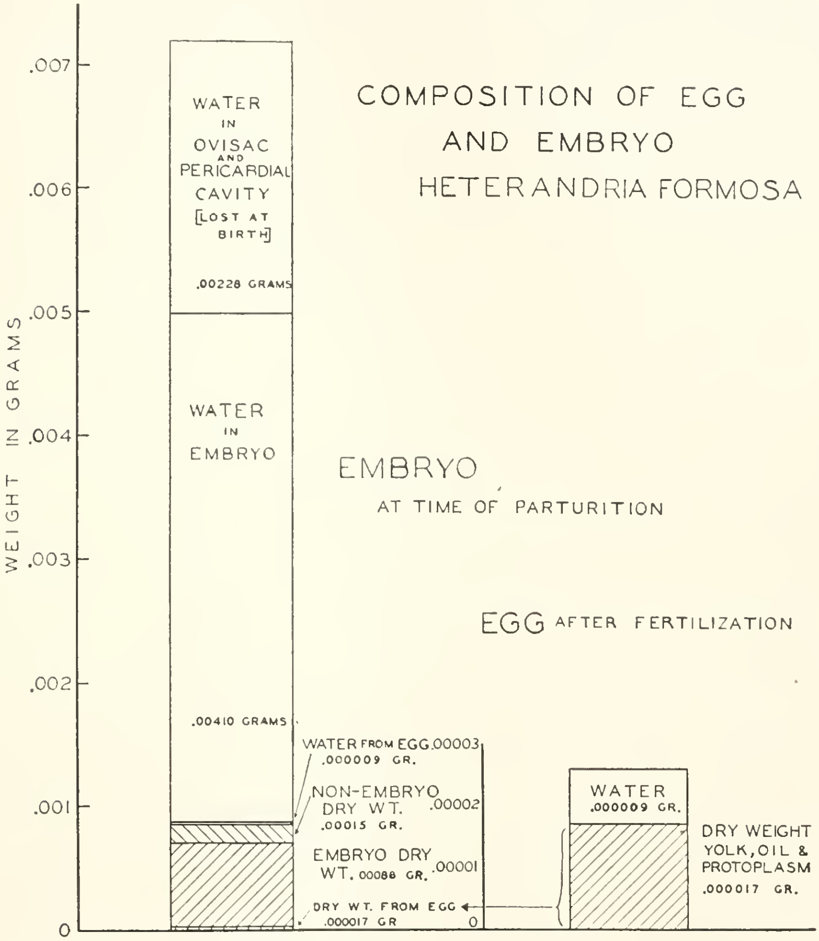

Figure 8. The composition of a typical embryo ready for parturition and of a recently fertilized egg is diagrammed. Wide variations from the actual figures cited were found but the proportions remained generally the same in the various larvae studied. As discussed in the text, the weight figures and the relative amounts of wet and dry material in the fertilized egg have been determined only approximately.

In the above figures for the trout the discrepancy of .14 grams between the 1.7 grams of yolk and water and the resulting 1.56 gram weight of the larva represents the dry weight used for maintenance metabolism. The dry weight used for maintenance in Heterandria could not be measured directly. However, it can be assumed that approximately one-third of the total food available is used for the maintenance of the embryo in Heterandria as well as in the trout.[4] The maintenance dry weight calculated in this way can be introduced into an equation expressing the actual dry weight drain on the mother per embryo.

$$\text{water} + \underset{\substack{\text{(dry weight}\\ \text{of embryo)}}}{6.8\text{ mg.}} + \underset{\substack{\text{(maintenance}\\ \text{dry weight)}}}{2.2\text{ mg.}} = \underset{\substack{\text{(total dry}\\ \text{weight from}\\ \text{mother)}}}{9.0\text{ mg.}} + \text{water}$$

Growth Rate of the Developing Embryo

Thus far the magnitude of the embryonic weight increase has been described, but no indication of the rate has been obtained. Observations of living gravid females suggested a method whereby a time axis might be obtained. The young of females kept under constant conditions showed a tendency to be born at regular intervals.[5] Accordingly, the weights of all the broods of a single female kept under relatively constant conditions were plotted as if the time intervals between them were the same. This treatment yielded consistent and apparently significant growth curves.

The female whose embryos are presented in Figure 9 has the unusually large number of eight broods. The growth relationships believed to be general for Heterandria are therefore well shown. The initial portion of the curve is a straight line which appears to be parallel to the time axis. This line represents the early developmental period when no post-fertilization contribution of nourishment from the mother can be detected. The second part of the curve is a steeply ascending straight line. It extends throughout the greater part of the embryonic period and represents an approximately constant growth increment in dry weight contributed by the mother. Obviously, there must be a transitional period, which the data cannot show, during which the pseudo-placental associations are being established. This has been indicated by the dotted lines in Figure 9 and also in Figure 10 which represents the similar treatment of embryo weights obtained in the study of other females. All of the females which had been kept under relatively constant conditions showed this relationship. Unfortunately, the concept cannot be tested for females with fewer than five broods as can be seen from the number of points required to establish the two straight lines in Figures 9 and 10.

In order to extend the study to more females, records of ovaries dissected over a three year period were re-examined. These records gave the number of broods per female together with their average ovisac diameters for fish kept under a variety of experimental conditions. The average weight of the broods in these females

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[4] The ratio between the dry weight of the embryo and the dry weight of nutriment required to produce this quantity of embryo is the efficiency coefficient of development. Gray reports a value of .65 for the trout and points out that this figure has been found to be approximately the same for a wide variety of organisms (cf. Murray, 1926, chick; Hayes, 1930, Atlantic salmon; etc.).

[5] Unpublished data. It is difficult to obtain precise records because the young are frequently eaten by the parent and are so small as to be easily missed. Rather constant optimal conditions and mature females in good health are required to demonstrate this. Small females will not show it clearly. On the whole it is remarkable that this tendency toward even spacing of the broods is as frequent and demonstrable as the data suggest it to be.

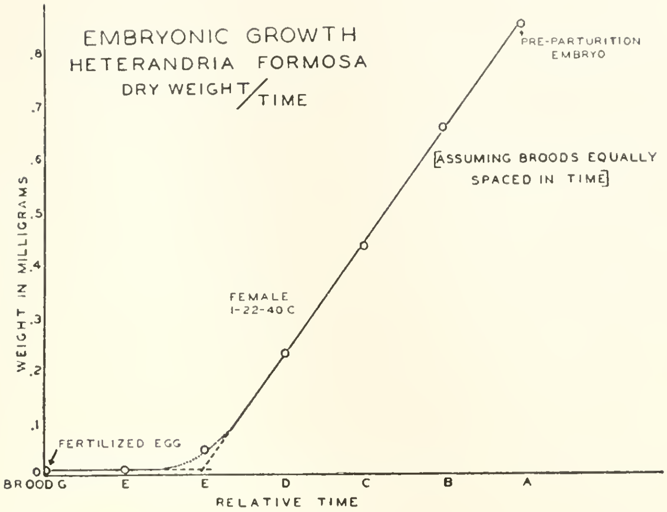


FIGURE 9. The average dry weight of the embryos in each brood of a female with eight fertilized broods is plotted as a function of time. Since the actual age of the embryos cannot be determined, no specific time units can be employed. However, in any large, mature female of Heterandria kept under reasonably constant conditions the broods of embryos seem to be born at approximately equal intervals. Therefore, the broods of embryos still in the ovary can be equally spaced on a time axis without the necessity of specifying the actual number of days or hours between them. Since the time relations between the various broods contained in the ovary are thus represented without using known time units, a relative time scale is actually employed. This concept of relative time is also used in Figures 10–12 and throughout the text. The female has been kept under constant illumination in a room with only slight variations in temperature.

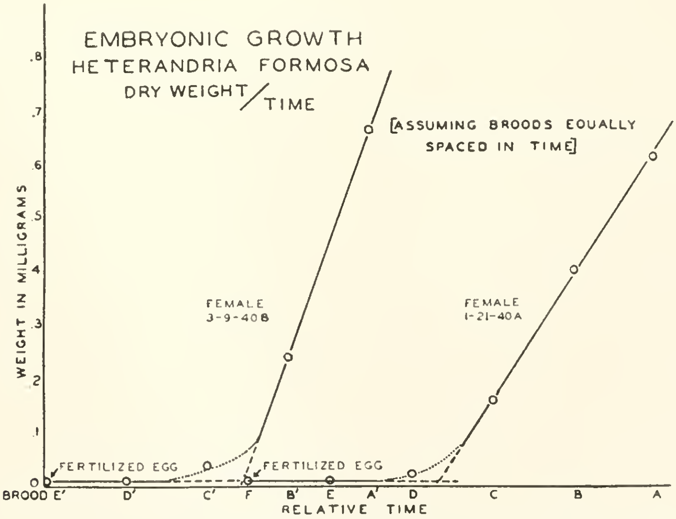


FIGURE 10. The average dry weight of the embryos in each brood of two different females, one with five and the other with nine broods, is plotted as a function of relative time. The treatment is the same as in Figure 9.

could be estimated by comparison of the ovisac diameters with the average weight of the embryos as shown in Figure 7. The results were plotted exactly as in the above cases where the weights were obtained directly. Despite the wide variety of ages and conditions represented, the majority showed the generally linear nature of growth in the two periods. Data from three such females are presented in Figure 11.

This even spacing can also be demonstrated by the superposition of growth curves from different females. Numerous females were examined by this method and their intermediate broods were found to fall on the growth curves of other females. For example, Figure 9 shows the growth curve for the embryos of a female with eight broods. Broods B and E of this female (second and fifth broods respectively) were found to correspond in weight to the first and third broods of a four-brooded female. When the second brood of the four-brooded female was arbitrarily placed midway between broods B and E on the time axis, it was found to have a weight value which placed it on the growth curve representing the embryos of the eight-brooded fish.

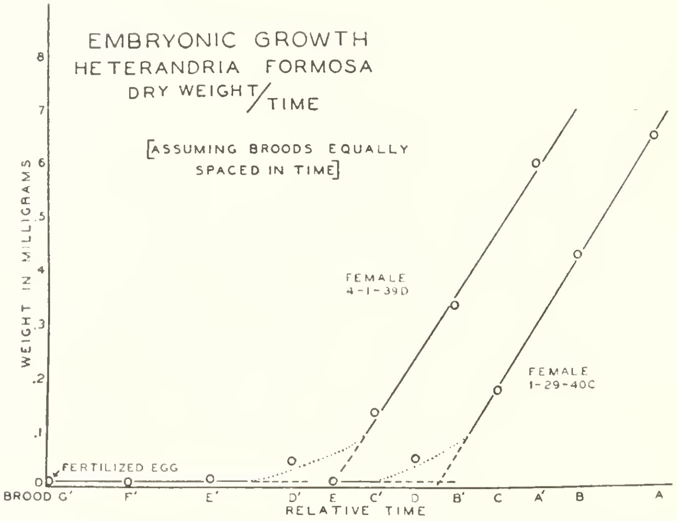
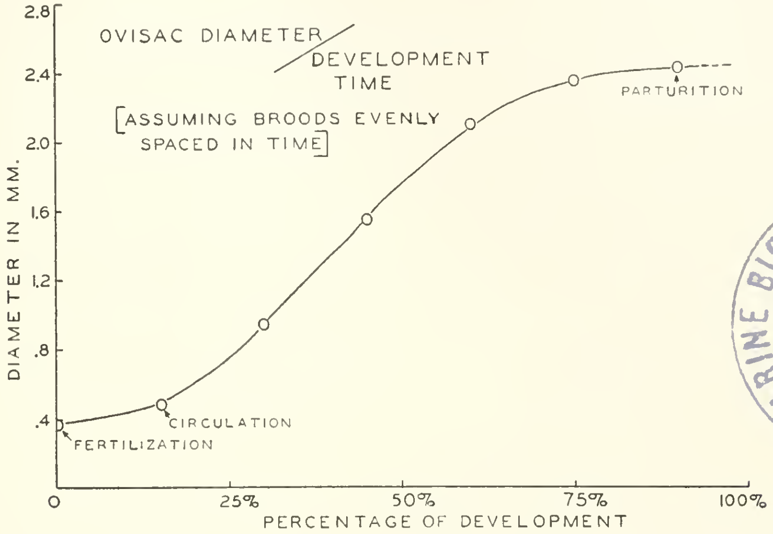


FIGURE 11. The average dry weight of the embryos in each brood of two different females, one with seven and the other with five broods, is plotted as a function of relative time. The dry weight values have been obtained by use of the ovisac diameter and the dry weight curve of Figure 7. The broods are assumed to be evenly spaced in time.

The diameter of the ovisac for each brood of an eight-brooded female can itself be plotted as a function of relative time (Fig. 12). The resulting curve is smooth and sigmoid, showing a slower diameter increase during the early and late periods. In the female represented in Figure 12 the earliest brood was observed to represent recently fertilized ova, and the latest brood was found to represent embryos ready for parturition. Hence a point midway on the relative time axis should indicate the ovisac diameter of an embryo roughly half way through development. It is possible in this fashion to determine the percentage of development represented by other ovisac diameters.

In the course of these studies on Heterandria the specific stage of development corresponding to various ovisac diameters has been noted. It is now possible to assign the percentages of development determined for certain ovisac diameters to developmental stages in Heterandria. These can then be compared with similar stages described in closely related fish in which a more direct measure of time can be obtained. Bailey (1933) faced with similar difficulties in determining the exact time of development in the poeciliid *Xiphophorus helleri*, staged 50 ova and embryos and selected the tenth, twentieth, thirtieth, etc. as representing corresponding percentages of development. *Fundulus heteroclitus* was studied at the Marine Biological Laboratory, Woods Hole, in July 1939 and together with Xiphophorus was compared with Heterandria. The relative times between common stages were clearly similar in the three species. Although the comparison could be only approximate,[6] it reduced the likelihood of a serious error in the time relations assigned to Heterandria.



MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY
MASS.

FIGURE 12. The average ovisac diameter of each brood in a female with eight broods is plotted as a function of percentage of development. This is the same female shown in Figure 6 and the time units are obtained in the same manner. However, since the entire range from the fertilized egg to the embryo ready for parturition is represented, these extremes are indicated as 0 per cent and 100 per cent of development and the relative time axis is subdivided accordingly.

If these time relationships are used to determine the increments of growth per unit of time during the later embryonic period, it will be seen that the increments remain constant. When the percentage of maximum increment of dry weight is plotted as function of time in Heterandria (Fig. 13), the result is a straight line parallel to the time axis throughout most of the embryonic period. Gray, treating his growth rate data for the trout in this manner, demonstrated an asymmetrical rise and fall (Fig. 13). This represented a deviation from Robertson's formula (1923) in which the percentage of maximum increment plotted as a function of the size of the embryo shows a symmetrical rise and fall. This difference Gray was able to explain by demonstrating that the growth rate is also a function of the yolk remaining in the yolk sac.

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[6] In addition to the difficulties of comparing stages described for different species, the relative time between embryonic stages may be changed by exposure to different temperatures. At the same temperature the relative time between similar stages in two different species may be different (Moore, 1939; Worley, 1933).

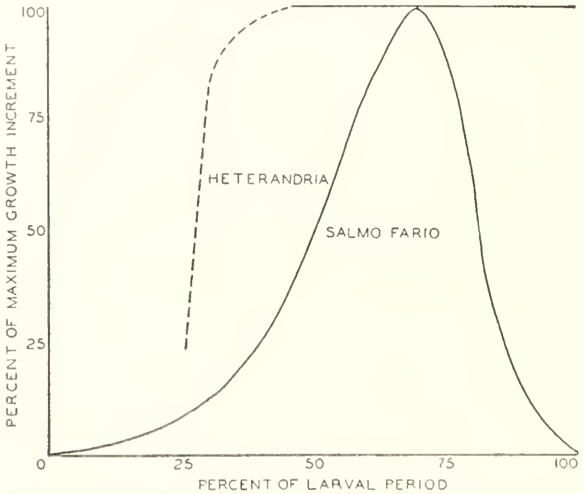

Figure 13. The percentage of maximum increment of embryonic growth is plotted as a function of the per cent of the larval period for both *Heterandria formosa* and Salmo fario. No measurements are available for the early stages of Heterandria and the growth increments are necessarily inferred. The curve for Salmo also represents the product of the dry weight of the embryo times the dry weight of the remaining yolk plotted as a function of the per cent of larval development.

Discussion

The fact that *Heterandria formosa* represents the development of a true viviparity in which nearly all of the nourishment for embryonic development comes from the mother is of interest in itself. When the assumption of equal spacing of broods is made and the data treated accordingly, it also appears that the nourishment for the growth process is being used by the embryo at a constant rate. If this is true, it suggests that some specific extrinsic factor or factors is limiting embryonic growth in this species. Restricted food supply and limited oxygen availability were considered likely to affect the growth rate in this manner. The discussion which follows is an attempt to evaluate these two factors.

It has been shown that morphogenesis in the oviparous fish adjusts itself to the amount of nourishment available, i.e. that the size of the larva is dependent on the amount of yolk available (Morgan, 1896) and not on the total amount of cytoplasm (Sumner, 1900; Hoadley, 1928). That the rate of growth of the oviparous fish embryo is dependent not only on the mass of the embryo but also on the actual amount of yolk remaining has been shown by Gray (1926; 1928a, b) (cf. Fig. 12).

It is conceivable that the pseudo-placental barrier itself may increase in effectiveness only enough to allow for the steadily increasing demands of the maintenance metabolism. Such a relationship seems rather remarkable under the circumstances and might be expected to break down with the complications of retraction of the pericardial sac and development of special secretory cells in the follicle wall. No alteration in growth rate can be detected when these changes in the pseudo-placental barrier occur.

Instead of the limitation lying in this barrier, it may be that certain of the raw materials for growth are present in the maternal blood stream in limited amounts. How such a limitation could affect all of the broods in a similar manner is not clear. There is, however, some indirect evidence that the growth of the individual embryo is responsive to changes in the total maternal supply of nourishment available to all the embryos.

This evidence involves the young of fish not heavily burdened with embryos. Since the straight line nature of the growth curve cannot be tested unless more than four broods are present, all of the data used to develop the idea of a constant increment of growth have been obtained from females with many broods. In these the food requirements of the embryo must constitute a great drain on the mother. The physiological drain would not be as great in females which are recovering from unfavorable conditions, because they contain fewer broods than they are capable of supporting. In these females more nourishment per embryo should be available than in females which have been kept under relatively constant conditions. If more nourishment is available, the embryos should be larger at the end of the larval period (cf. Gray, 1928a). The actual results in Heterandria are that the first young born of a female recovering from unfavorable conditions are large.[7] This constitutes the best available evidence that the food supply of the embryos in a many brooded female is restricted in some manner, and supports the hypothesis that during the main growth period food may be the determining factor in the development of Heterandria at normal temperatures.

There is considerable evidence that at higher temperatures the oxygen supply to the embryo may be a factor limiting growth. According to Gray, all of the oxygen used by the trout embryo is for maintenance metabolism, the amount used for growth being almost negligible. He also found that a large drop in the growth rate of the trout caused no corresponding drop in oxygen consumption. Nevertheless, Jacques Loeb showed (1894) in Fundulus that development is directly retarded by lack of oxygen. The Heterandria females may under certain conditions at 25° temperature give birth to young regularly every four days. A rise in temperature of two or three degrees during the daytime for even two or three days may delay the next brood.[8] It seems likely that the decreased oxygen supply may account for

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[7] The following figures will not be discussed in detail but are presented in support of the statements made above. A female recovering from unfavorable conditions was observed to give birth to young on Dec. 13 and 14 after a lapse of several months. These young were very much larger than those born four and ten days later. The actual dry weights were found to be:

Dec. 13 and 14, 1940	Dec. 18 and 26, 1940
.88 mg.	.63 mg.
.95 mg.	.64 mg.
.89 mg.	.61 mg.

[8] Scrimshaw—unpublished data.

this. At the higher temperatures both the mother and the embryos require more oxygen per unit time, but less oxygen is dissolved in the water. It also seems likely (cf. Irving, 1941) that the oxygen dissociation curve of the hemoglobin in the blood would be shifted to the right and flattened, and as a result the oxygen carrying power of the blood would be reduced by the increased temperature.

It can also be observed that a female kept at a constant temperature of 25° C. will have a number of its embryos dying within several hours when the temperature is raised to 28° C.[9] Such a temperature is not in itself supra-maximal, for the temperature tolerance of the mother runs well above 34° C. The young after birth grow well at this high temperature. The embryos at all stages of development seem to tolerate this temperature satisfactorily when isolated from the mother if the water is well aerated. For example, their heart rate shows no irregularity in μ value on an Arrhenius plot until the temperature reaches 34.6° C. The death of the embryos in the above case can be explained on the basis of limited oxygen supply to them.

Oxygen supply is not likely to be the limiting factor at ordinary temperatures. When exposed only to natural daylight, a female kept at a constant temperature and under approximately uniform feeding conditions will contain a certain number of embryos and these will show the constant growth increment described. When such a female, other conditions remaining the same, is exposed to continuous artificial light for about a month, the number of embryos markedly increases.[10] There is no reason to believe that the total availability of oxygen has significantly increased.

It seems reasonable to believe that the oxygen is the principal limiting factor at higher temperatures, and food supply at the moderate ones. This would mean an intermediate range in which the two factors are complementary in a regular fashion. The data do not serve to distinguish between different degrees of limitation. Furthermore, Heterandria kept at temperatures high enough to limit the oxygen supply do not have enough broods to enable the growth rate to be determined. The possibility of other factors such as endocrine balance influencing the growth rate has not been excluded.

SUMMARY

The fertilized egg of the viviparous poeciliid *Heterandria formosa* is minute and is made up almost entirely of a single large oil globule. At least 70 per cent of the original volume of the oil globule is still present when maternal contribution of nourishment begins. Secretory cells develop in the ovisac wall late in the embryonic period. These together with adaptations previously described permit the mother to contribute nearly all of the raw materials for growth and development of the embryo after the egg has been fertilized.

The increase in wet and dry weight of the embryos at the expense of the mother has been determined by obtaining both wet and dry weights at various stages of development. The dry weight of the embryo increases from 0.017 milligrams at the time of fertilization to 6.8 milligrams at the time of parturition. The percentage dry weight remains constant at 13.2 per cent after the pseudo-placental association is established.

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[9] Scrimshaw—unpublished data.

[10] Scrimshaw—unpublished data.

Observations of living females suggested that under constant optimal conditions the broods of a single female tend to be evenly spaced in time. Upon this assumption the dry weights of the embryos in each brood of suitable females were plotted against relative age. The resulting curves suggested that the rate of growth after the maternal contribution of nourishment can be detected is approximately constant. Food supply and oxygen supply are discussed as factors which might limit this growth. Embryonic growth in Heterandria is compared with that in oviparous fishes.

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THE CAPILLARY BED OF THE CENTRAL NERVOUS SYSTEM OF CERTAIN INVERTEBRATES

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(From the Department of Anatomy, Western Reserve University School of Medicine, Cleveland, and the Marine Biological Laboratory, Woods Hole) [1]

The vascular pattern of the vertebrate brain may be either one of two types: the one consists of single vessels that anastomose to form a continuous capillary network; the other consists of paired vessels that end in capillary loops. These two types are, as a rule, mutually exclusive, except in the lungfish, Epiceratodus (Craigie, 1943), and in the salamander, Ambystoma (Craigie, 1938a), where both networks and loops occur. The network pattern is the more common type. It is found in monotremes (Sunderland, 1941) and in all placental mammals, in reptiles with the exception of the lizards, in anuran amphibians, and in the fishes including the hagfish, Myxine. The paired vessels ending in capillary loops are characteristic of the marsupials (Wislocki and Campell, 1937; Craigie, 1938b; Sunderland, 1941), the lizards (Schöbl, 1878; Sterzi, 1904) including Sphenodon (Craigie, 1941a), and the tailed amphibians (Schöbl, 1882; Sterzi, 1904; Craigie, 1938a; 1939; 1940a) including the Gymnophiona (Craigie, 1940b; 1941b). The brain of the lamprey, Petromyzon, is also supplied by loops (Craigie, 1938a).

The study of patterns of cerebral vascularization has been extended here to include invertebrates. In most invertebrates blood vessels do not enter the nervous tissue. There are, however, exceptions. Havet (1916), for instance, in his investigation of the glia cells of the invertebrates mentions the existence of blood vessels within the central nervous system of the earthworm. Another reference may be found in Cajal's (1929) paper on the origin of unipolar neurons in invertebrates according to which the cerebral ganglia of the squid are vascularized by intraganglionic blood vessels.[2] Both of these animals, the earthworm and the squid, have been studied, therefore, and the blood vessels supplying their ganglia have been compared with those of vertebrates.

Material and Methods

Large earthworms (*Lumbricus terrestris* [3]) were collected on lawns in the Cleveland area during rainy nights and were fixed with Zenker-formol. The ring consisting of cerebral and subesophageal ganglia and their connectives was embedded in paraffin and cut 7 micra thick. The sections were stained with Masson's

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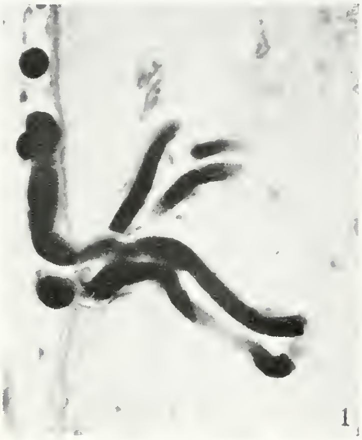
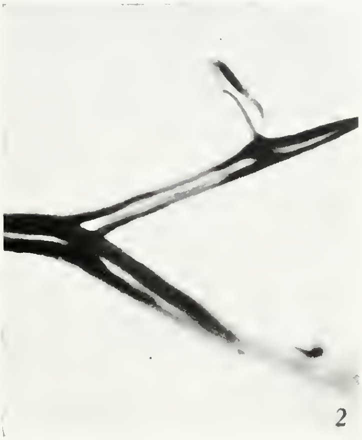
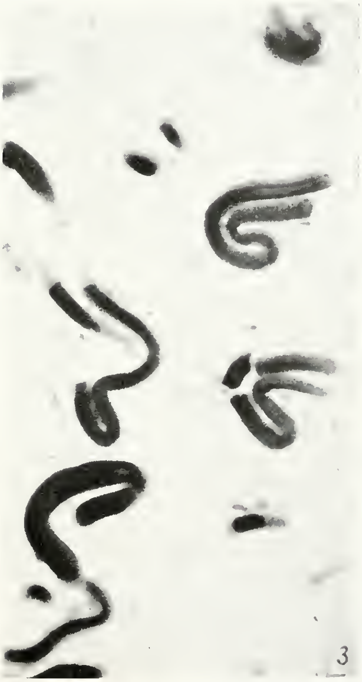
[1] This research was aided by a grant made to Western Reserve University by the Rockefeller Foundation.

[2] The papers of Williams (1902) and Grimpe (1913) give excellent accounts of the vascular system of cephalopodes, but do not include descriptions of the vascularization of the cerebral ganglia.

[3] *Lumbricus terrestris* was introduced from Europe and has become widely distributed in Ohio in the past 25 years (Eaton, 1942).







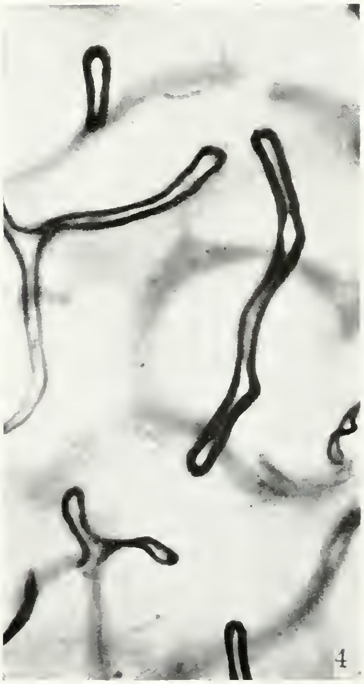


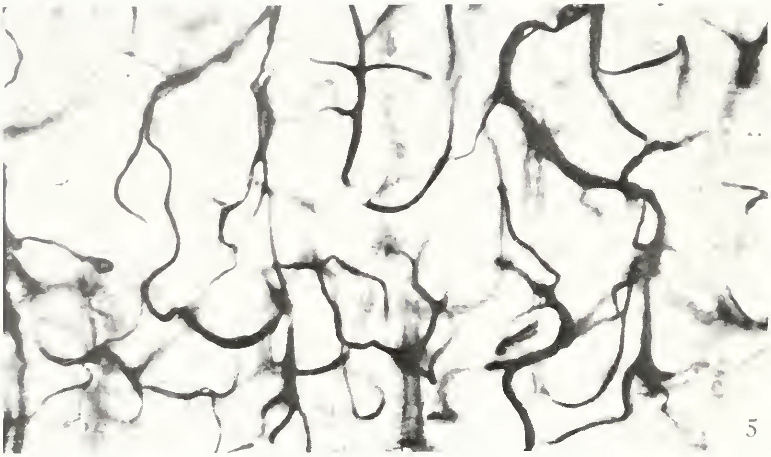
FIGURE 1. A pair of branching blood vessels in the central nervous system of the earthworm. Zenker-formol, paraffin, 7 micra, Masson's trichrome stain. Photomicrograph, × 350.

FIGURE 2. A pair of blood vessels in the brain of the opossum branching in the same manner as those of the earthworm shown in Figure 1. Injection with India ink-gelatin, formalin, nitrocellulose, 100 micra. Photomicrograph, × 350.

FIGURE 3. Terminal loops in the central nervous system of the earthworm. Technique and magnification as in Figure 1.

FIGURE 4. Terminal loops in the brain of the opossum. Technique and magnification as in Figure 2.

trichrome stain. Probably because of the strong contraction of the animals when the fixing fluid is injected into the body cavity, the central ganglia sometimes become very hyperemic. In such animals the blood vessels of the central nervous system are filled with the blood fluid which stains well with the red component of the Masson stain (see Figs. 1 and 3).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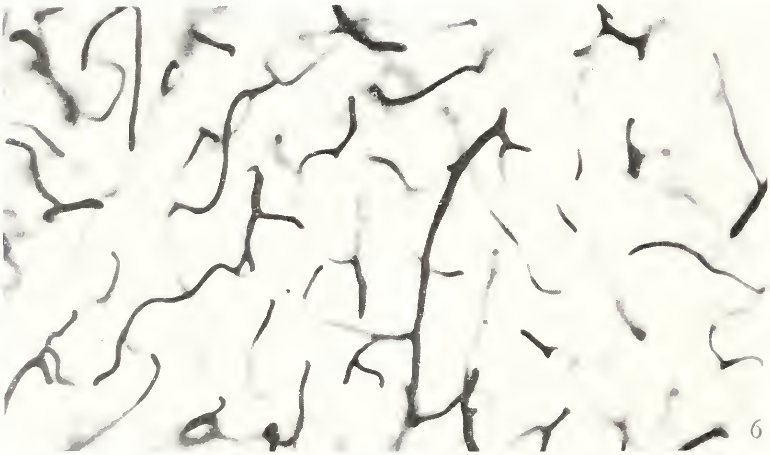
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FIGURE 5. Capillary network in the cerebral ganglion of the squid. Injection with India ink-gelatin, formalin, nitrocellulose, 100 micra. Photomicrograph, × 220.

FIGURE 6. Capillary network in the brain of the rat. Technique and magnification as in Figure 5.

Squids (*Logligo pealii*) were obtained at the Marine Biological Laboratory at Woods Hole and were injected with India ink-gelatin through the heart in the same manner as vertebrates. The injected cerebral ganglia were fixed in formalin, embedded in nitrocellulose, and sectioned 100 micra thick. The blood vessels observed in the central nervous system of both the earthworm and the squid were compared with those in various vertebrates, including monkey, cat, guinea pig, rat, opossum, alligator, and several species of teleosts. These were all injected with carmin- or India ink-gelatin, were embedded in nitrocellulose, and were sectioned 100 or 200 micra thick.

Observations

A comparison of the illustrations (Figs. 1 to 4) shows that the blood vessels of the central nervous system of the earthworm are of the same type as those of the opossum brain. In the earthworm blood vessels enter the tissue of the central nervous system in pairs. They divide together and their branches form corresponding pairs (Fig. 1). Finally the two limbs of each pair join and thus end in hairpin-like loops (Fig. 3). This is essentially the same arrangement which Wislocki and Campbell (1937) described in the opossum where arteries and veins stay together in pairs after they have entered the brain tissue. Whenever an artery divides, the accompanying vein divides the same way (Fig. 2), and all blood vessels within the opossum brain end finally in non-anastomosing loops (Fig. 4).

The vascular pattern of the central nervous system of the squid is entirely different from that of the earthworm. In the squid arteries and veins enter the cerebral ganglia singly. Their branches form a network of anastomosing capillaries (Fig. 5), just as in the brain of placental mammals (Fig. 6).

Discussion

"Since the discovery by Schöbl (1878) that there exist in reptiles two radically different types of cerebral vascular bed, one reticular and the other composed of independent, non-anastomosing capillary loops, the relationship between these two types has remained obscure and attempts to reconstruct the phylogenetic history of this mechanism have been complicated rather than simplified by increasing knowledge of the occurrence of the loop arrangement in various vertebrate classes" (Craigie, 1941a, p. 263). The difficulties inherent in the application of the phylogenetic concept to the cerebral vascular patterns are well illustrated by the fact that among the cyclostomes, a group of primitive vertebrates, one (Petromyzon) shows loops, another (Myxine) a network (Craigie, 1938a). The description presented here of loops in the earthworm and of a network in the squid only serves to accentuate these difficulties.

An attempt is made here, therefore, to illustrate a common origin of both systems by dispensing with the phylogenetic aspect altogether. The loop system is not considered as a primitive forerunner of the network pattern, but is presented as the result of a parallel development capable of differentiation and functional efficiency corresponding to that of the reticular type.

The origin of the cerebral vascular system may be compared with that of the endocellular blood vessels in the large extramedullary nerve cells of certain fishes such as the swellfish, *Spheroides maculatus*. In young specimens each one of these cells is surrounded by a network of blood vessels. As the cells become larger in older animals the distance between the center of the cell and the blood vessels apparently becomes too great and the blood vessels enter the cytoplasm. Similarly the whole nervous system while still small could be vascularized by a network of superficial blood vessels (Fig. 7 AB). Such a condition actually obtains in the

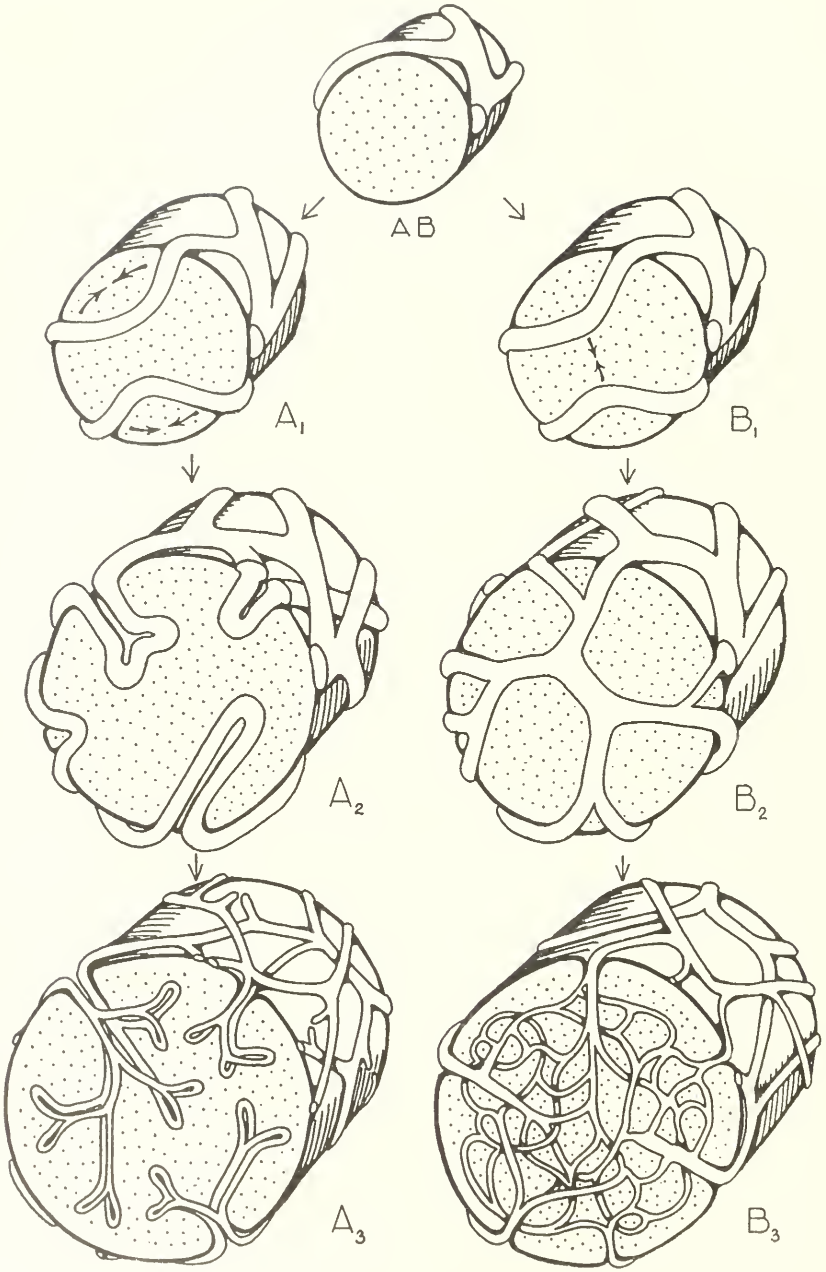

FIGURE 7. Diagram illustrating the derivation of loops and network patterns from a common origin. AB, primitive central nervous system vascularized by superficial network. A_1 and B_1, vessels of the superficial network come to lie within the nervous tissue. There are two possibilities of further development: the blood vessels approach each other in the direction of the arrows (A_1) and thus become paired, or they send out branches which anastomose (B_1). In the one case loops are formed (A_2), in the other a network results (B_2). Both these types are capable of further development to more complex systems (A_3 and B_3).

central nervous system of Amphioxus and in the spinal cord of Petromyzon which are vascularized by networks of superficial blood vessels. With the increase in size of the central nervous system segments of vessels forming part of the surface network come to lie within the nervous tissue (Figs. 7 A_1 and B_1). From this stage both the paired vessels ending in loops (Fig. 7 A_2) and the network (Fig. 7 B_2), may be derived as indicated. Both types occur in invertebrates and vertebrates, and both become eventually highly complex in mammals (Figs. 7 A_3 and B_3).

In this scheme the position of the animal in the phylogenetic order is not considered. This means that the step from A_1 to A_2 or from B_1 to B_2 can be taken anywhere within the vertebrates or the invertebrates. Thus the earthworm follows A_1 to A_2, the squid B_1 to B_2. Among the cyclostomes Petromyzon follows A_1 to A_2, Myxine B_1 to B_2. Epiceratodus and Ambystoma combine the two patterns, a situation which is not illustrated in Figure 7, but which can easily be visualized.

The question still remains: Which factors cause the cerebral blood vessels of the earthworm, of Petromyzon, of the opossum, etc. to differentiate as loops, and those of the squid, of Myxine, and of most vertebrates as a network. An answer to this question is to be expected from the study of the early development of cerebral blood vessels and from the application of the methods of experimental embryology.

Summary

The blood vessels supplying the central nervous system of the earthworm are of the same type as those in the brains of tailed amphibians, lizards, and marsupials, i.e. the blood vessels are paired and end in loops. The blood vessels in the cerebral ganglia of the squid form a network like that which occurs in the brains of fishes, anuran amphibians, reptiles (except lizards), birds, and placental mammals. The origin of both systems is discussed.

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BIOLOGY OF THE CALIFORNIA SEA-MUSSEL (MYTILUS CALIFORNIANUS). III. ENVIRONMENTAL CONDITIONS AND RATE OF GROWTH[1]

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In the cultivation of oysters, clams and other mollusks, as well as in studies on their rates of growth under natural conditions, it has been frequently observed that certain years are more favorable than others for rapid increment in size. But there has been no satisfactory analysis of the environmental conditions responsible for the observed differences in growth rates. For this reason an experimental study, extending over four years, has been made of the growth of the California sea-mussel at the pier of the Scripps Institution of Oceanography. At this station daily records are made of the temperature of the water and of the numbers of dinoflagellates, diatoms and bacteria present and potentially contributory to the mussels' ultimate food supply. For the temperature records the writers are indebted to Capt. S. W. Chambers; for the data on phytoplankton to Prof. W. E. Allen, and for those on bacteria to Prof. C. E. ZoBell. They also appreciate the technical assistance of Miss Harriet Dunn and Mr. Carl Johnson.

In two previous papers (Coe and Fox, 1942; Fox and Coe, 1943) the writers have presented evidence relative to the normal rates of growth in this species at different seasons and at different ages and sizes, the different rates in the two sexes, the nature of the food materials and the influence of environmental conditions on the rates of growth. It was concluded from these observations that there is a generally positive correlation of the growth rates both with temperature changes and with the abundance of dinoflagellates present in the water. No similar correlation was found relative to the numbers of diatoms or bacteria. It was emphasized however that the correspondence between the size of the dinoflagellate populations and the growth rates of the mussels was not to be interpreted as the direct effect of dinoflagellates as potential food material, since a large proportion of the living dinoflagellates ingested usually pass apparently unchanged through the mussel's digestive tract. Furthermore the total supply of living phytoplankton which the mussel could possibly obtain is estimated to be so small in amount that even if all the constituents could be fully utilized they would furnish less than one-fifth of the mussels' nutritive requirements. The principal portion of the food was shown to consist of finely divided organic detritus, derived from the disintegration of many kinds of marine animals and plants, including both unicellular and multicellular forms (Fox and Coe, 1943).

Continuation of those observations during three additional years has shown that there are wide variations in the mussels' growth rates, not only from month to month but also from year to year. It is the object of this paper to record these variations and particularly to present such evidence as has now been obtained as to their causes. For this purpose the changes in the environmental conditions from month to month have been analyzed as fully as possible. Since there are no rivers in the vicinity and the annual rainfall is small, there is but little variation in salinity. The principal effective variables are the temperature and the food supply.

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[1] Contributions from the Scripps Institution of Oceanography of the University of California, New Series No. 233.

The experimental mussels were kept in wire-screened boxes immersed in the sea below the low-tide level. Since the increment in size was found to vary with the age of the individual under identical environmental conditions (Coe and Fox, 1942), it was necessary to have the same ages represented at all times. This required the addition of young individuals from month to month and the removal of the oldest.

The experiment was continued from January 1940, to January 1944, with the exception of the first five months of 1942. At nearly all times the experimental boxes contained from 100 to 400 or more individuals, each age group being in a separate compartment. The average age remained nearly constant and all were sexually immature. When the individuals of a group were separately numbered, it was found that some grew rapidly for a period and were then overtaken by others; some became leaders for several months, while others remained dwarfs. For statistical purposes it was therefore desirable to follow the growth of 20 or more individuals of each age group. The mean monthly increments in size for all groups are shown in Figures 1 to 4.

In any consideration of the environmental conditions, it must be kept in mind that these conditions are constantly changing, due to the water currents that continually sweep past the pier at rates averaging from four to five miles per day. Consequently these conditions may vary considerably from day to day and even from hour to hour. The monthly means, however, will give a reasonably close approximation to the prevailing environments.

A comparison of the graphs in Figures 1 to 4 shows that the growth rates of the mussels have varied considerably, not only from year to year but also for the corresponding months of the years. The mean monthly increment in length for all groups was 3.43 mm. in the year 1940, 3.96 mm. in 1941, 5.43 mm. during the last seven months of 1942 and 5.11 mm. in 1943. It is evident from these figures that 1942 and 1943 were more favorable for rapid growth than either of the other two years and that 1940 was the least favorable. The lowest rate for any month of the four years was in August 1940. During that month the mean increment in length was only 1.6 mm., which was less than one-third as great as in the corresponding month of each of the three other years. The maximum rate occurred during April in 1940, during June in 1941, during July in 1942 and during May and July in 1943 (Figs. 1–4).

An examination of the environmental conditions, particularly as concerns temperature, storms and abundance of the phytoplankton during these years, will give some indication of the influence of each on the observed growth rates of the mussels.

First Year, 1940

The monthly growth rates of the mussels during this year were exceptional in that they showed fewer positive correlations with the temperature and with the abundance of dinoflagellates than in any of the other years. Following a decrease in the rate during February there was a rapid increase to a maximum in May, fol-

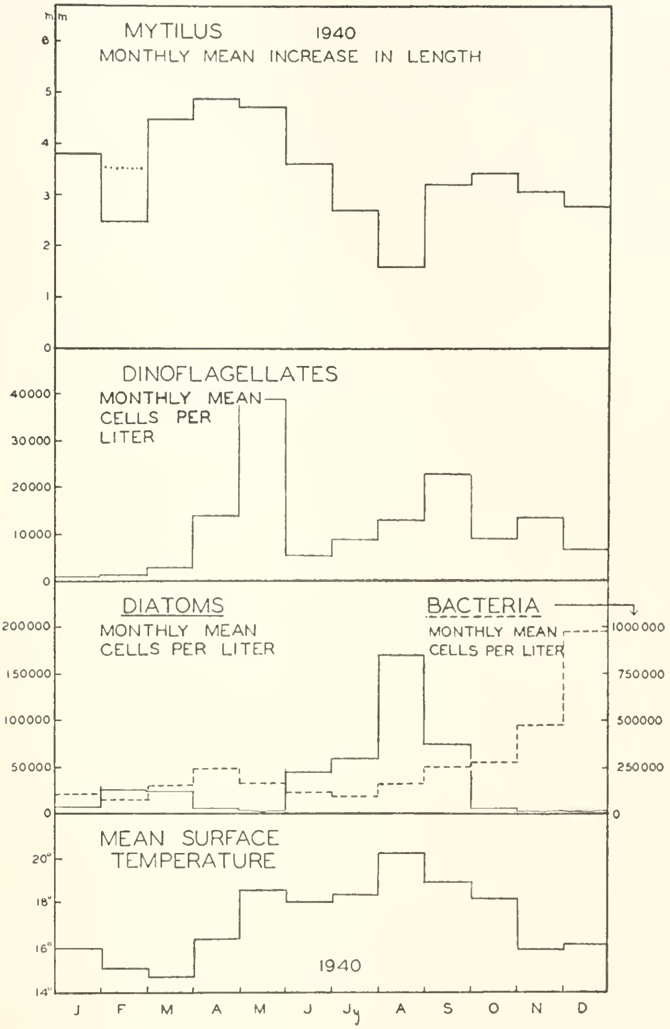

FIGURE 1. Graphs showing the average monthly growth rate of 453 mussels, divided into 11 groups according to age, and the abundance of dinoflagellates, diatoms and bacteria, as well as the average monthly temperature of the water during the year 1940. The depression of the growth rate in February was mainly due to a reduction of the feeding period to 22 days because of accidents caused by storms; the dotted line indicates the estimated increment if the accidents had not occurred. It was necessary also to estimate the growth in December because of an accident due to storm. The numbers of dinoflagellates shown in the graph differ in several cases from those indicated in Figure 4 of our previous paper (1942) because of erroneous data supplied to us at that time.

With some exceptions the growth rates were highest during those months having large dinoflagellate populations and in which the temperature exceeded 16° C.

lowed by a continuous decline to the lowest rate for any month of the four years in August (Fig. 1). The only explanation that can now be given for this exceptionally low rate in August is that for some unknown reason the organic detritus which furnishes the greater part of the mussel's nutrition was not present in sufficient quantity. The sharp drop in February was in part due to storms which necessitated removing the mussels from the sea and keeping them in the aquarium for six days. Since no increase in size occurs in the aquarium except when additional food is supplied (Coe and Fox, 1942), there was a possible feeding period of only 22 days during that month. Computed on the basis of the growth during that period, the estimated increase per day in February would be but slightly less than during the preceding month, as indicated by the dotted line in the graph (Fig. 1). A sharp rise in the growth rate in September and an additional increase in October was followed by the usual decline during the last two months of the year.

As a general rule, but with some conspicuous exceptions, the most rapid increment in size occurred during those months in which a large population of dinoflagellates was present and in which the temperature exceeded 16° C. Neither the diatoms nor the bacteria showed definite correlations with the growth rates of the mussels (Fig. 1).

Second Year, 1941

During the second year the growth rate was somewhat higher than in the preceding year, although the average number of dinoflagellates was smaller and the diatoms were less than half as abundant as in 1940. With the exception of February there was a continuous rise in the growth rate to a maximum in June, with a steady decrease thereafter (Fig. 2). The dinoflagellate population correspondingly reached a maximum in July, followed by a continuous decline to a minimum in December. Neither the diatoms nor the bacteria showed similar trends. The rate indicated for December is lower than it would have been except for a severe storm which allowed a feeding period of only 27 days.

Third Year, 1942

The experiment was interrupted for the first five months of 1942, but the last seven months of the year showed a greater increment in growth than in the corresponding months of any of the other years. The maximum rate occurred in July, followed by a continuous decrease during the rest of the year, with the exception of a slight rise in November, followed by a small decrease in December (Fig. 3). The water during those months contained an average of more than five times as many dinoflagellates as in the last seven months of the preceding year and the average monthly increment in the lengths of the mussels was 5.4 mm. as compared with 4.3 mm. in the corresponding period of 1941. The average number of diatoms was smaller than in any of the other three years.

Fourth Year, 1943

During the year 1943 the average monthly increment in size was considerably greater than in 1940 or 1941 but somewhat less than in 1942 (Fig. 4). By comparing the rate for December 1942 (Fig. 3), with that of January 1943, it will be seen that a sharp drop occurred during the latter month. The cause of this decrease in growth rate may have been due to a severe storm which necessitated transferring the experimental box from the sea to the aquarium, where it remained for four days.

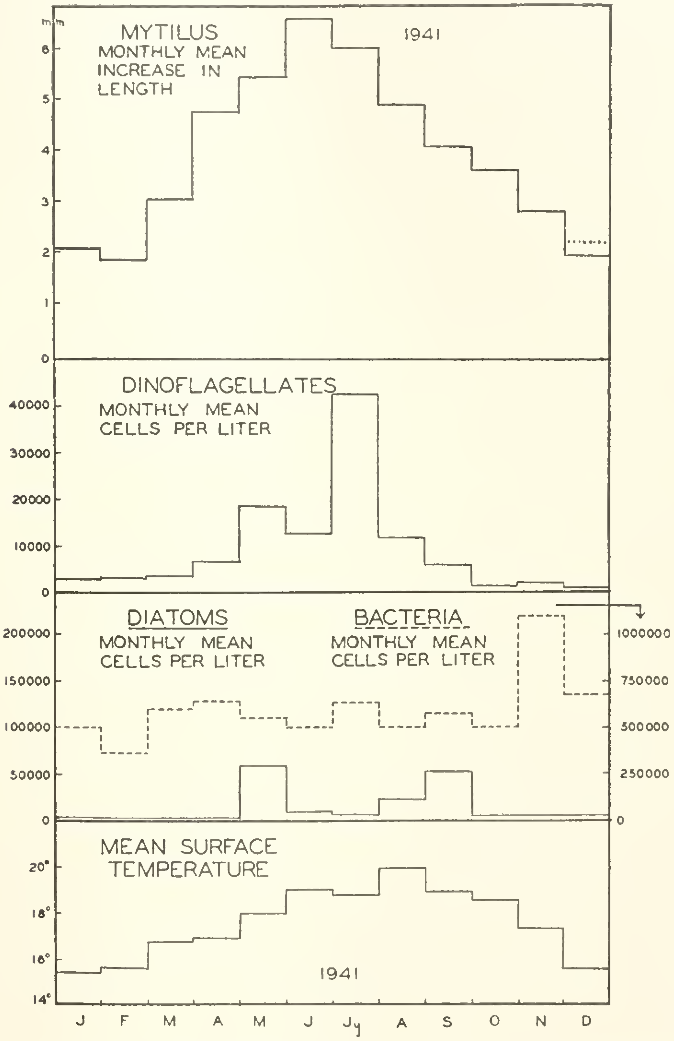

FIGURE 2. Graphs showing average monthly growth rate of mussels and abundance of dinoflagellates, diatoms and bacteria, as well as average monthly temperature of the water during the year 1941. The growth indicated for December represents a feeding period of only 27 days; the dotted line indicates the computed increase for a month of 31 days.

The most rapid increment in size occurred in those months having large populations of dinoflagellates, accompanied, presumably, by an abundance of organic detritus.

From this depression in January, the growth rate increased continuously until May, when the average increment was 6.4 mm. (Fig. 4). An unaccountable drop in the growth rate during June was followed by an average increment of 6.6 mm.

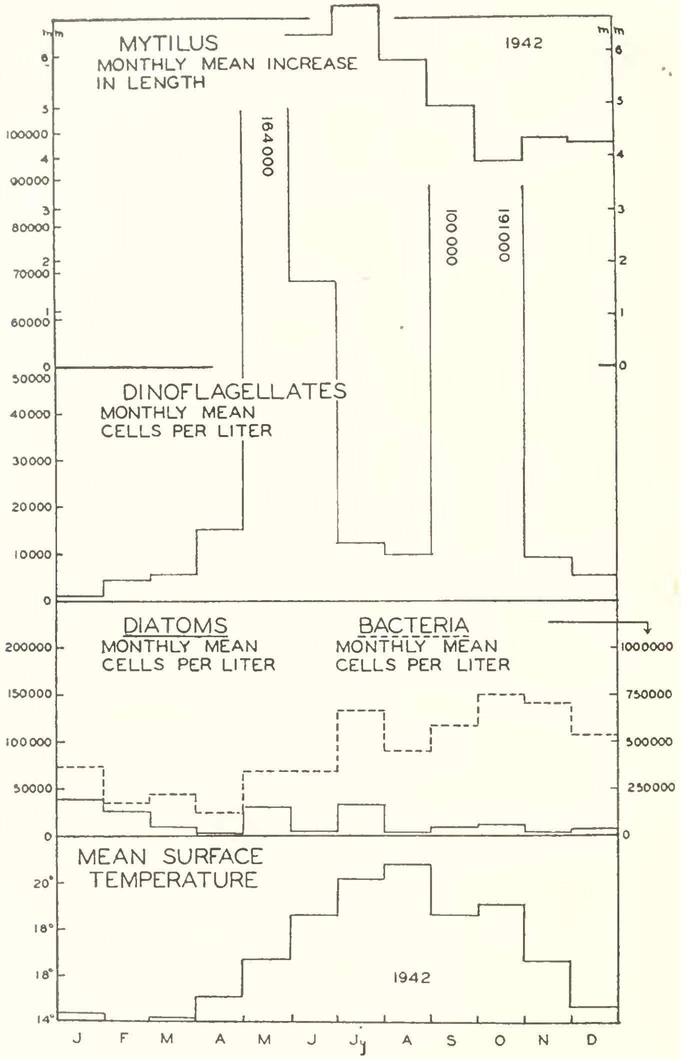

Figure 3. Graphs showing the mean monthly growth of mussels, the mean monthly temperature of the water and the mean monthly abundance of dinoflagellates, diatoms and bacteria during 1942.

in July, which was the highest rate for the year. Following the usual decrease in August, the rate continued high and steady during the two succeeding months; then, instead of the usual decline in November, there was a rise to an average of 6 mm., as compared with about 3 mm. in the corresponding month of 1940, 2 mm.

in 1941 and 4.3 mm. in 1942. In December the rate was but little more than half as great as during the preceding month (Fig. 4).

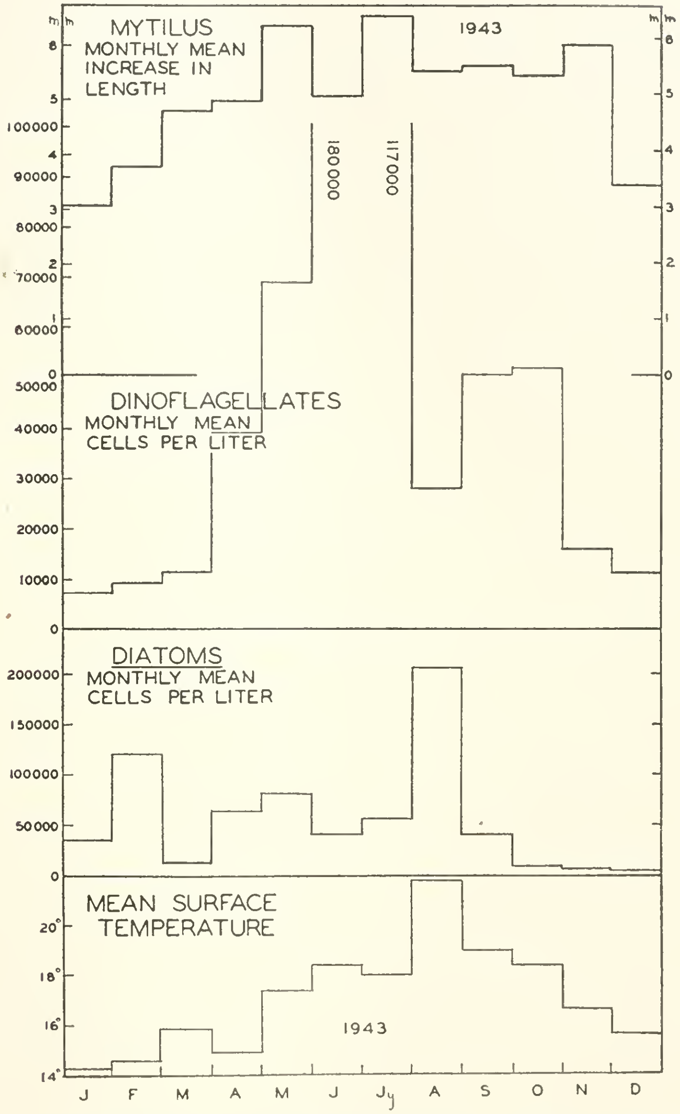

FIGURE 4. Graphs showing the mean monthly growth of mussels, the mean monthly temperature of the water and the mean monthly abundance of dinoflagellates and diatoms during the year 1943.

The average monthly increment in length was 5.1 mm. as compared with 3.43 mm. in 1940 and 3.96 mm. in 1941. This increased rate of growth was accompanied by populations of dinoflagellates more than four times as great as in either

1940 or 1941. The diatoms were also four times as numerous as in 1942, but again it should be emphasized that neither the dinoflagellates nor the diatoms furnish more than a small proportion of the food supply of the mussel.

Environmental Influences

The observations described on the foregoing pages show how great a variation was found in the growth rates of the mussels in different years and in different months of each year. The comparisons of these rates with the temperature and with the abundance of dinoflagellates, diatoms and bacteria are shown in Figures 1 to 4. There are obviously many other environmental conditions which are constantly exerting their influence on the growth of the mussels. Some of these may be of great importance but they are so sporadic in their action or so difficult to measure that no precise evaluation of their influence has as yet been possible.

There is little variation in salinity throughout the year and there is often a correlation between the amount of oxygen and the relative abundance of phytoplankton.

1. *Temperature*

As a general rule the rate of growth in mollusks increases with the temperature to a certain optimum and then rapidly decreases. Consequently the annual increment in length is greater in southern than in more northern localities because of the longer season favorable for rapid growth. The observations of Weymouth, McMillan and Rich (1931) on Siliqua, of Newcombe (1936) on Mya, of Chamberlain (1931) on Lampsilis, of Orton (1926–27) on Cardium and of Coe (1938) on Ostrea support this conclusion. The size eventually reached by the individual however is commonly much greater in the north because of the greater length of life.

At the pier of the Scripps Institution of Oceanography, where the experiment was conducted, the variation in the mean monthly surface temperature of the water during the year seldom exceeds 8° C. Both the low point of about 14° in winter and the high of about 22° in summer are well within range of the normal activities of the mussel. The highest temperature recorded at any time during these four years was 22.9° C., in August 1943, and the lowest was 13.4° in January 1943. Consequently growth continues throughout the year in this locality, although the rate of increment in length is only about half as great in midwinter as it usually is in the early summer. This decreased rate in winter is presumably due both to a lower state of metabolic activity and a decreased supply of nutritive materials.

In *M. californianus*, as in *M. edulis* (Loosanoff, 1942), feeding continues at temperatures both lower and higher than the extremes mentioned in the preceding paragraph. Under experimental conditions the California mussel will secrete byssus threads, ingest food and discharge feces at temperatures as high as 24 to 26° C. and to a less extent at 27 to 28°. The individuals subjected to the highest of these temperatures however died within 5 to 7 days. The lowest temperature at which ingestion and fecal discharge were found to occur was 7 to 8°. Since these mussels were subjected to the temperatures mentioned without any period of acclimatization from an aquarium temperature of 15°, it is considered probable that the figures given do not represent the extreme range of the mussels' potential metabolic activities.

Experiments previously made in this laboratory indicate that the maximum rate of filtration and maximum oxygen consumption take place at about 20° C., with distinctly lower rates below 15°. A decreased growth rate has occurred in August, the month of highest temperature, in each of the four years. But in 1940 the decrease began in June and reached its lowest rate in August, while in 1941 the highest growth rate occurred in June and in 1942 in July. In 1943 maxima occurred in May and July. The variability in the growth rates at corresponding temperatures in different years indicates that the food supply is more influential than small variations in temperature in determining the rates of growth. The prevalence of storms and high seas common in winter are doubtless detrimental to active growth.

2. *Food*

The mussel is essentially a scavenger, utilizing as food not only small unicellular organisms and dissociated cells but also the particulate disintegration products of any of the animals and plants which die in the vicinity or similar products which are brought from a distance by currents. Even the bacteria which cause the decomposition may themselves be utilized as an additional source of nourishment (Coe and Fox, 1942; Fox and Coe, 1943).

The constituents of the ingested materials have been ascertained at frequent intervals by examination of the stomach and intestinal contents and of the feces. The substances most commonly present are finely divided organic detritus, dinoflagellates, diatoms, silicoflagellates and bacteria; also tintinnids, flagellates, ciliates and other protozoans, as well as algal cells and fragments, algal spores, spermatozoa and ova (including those of its own species), together with inorganic substances such as particles of sand and shells. At times the organic materials may be ingested in amounts much greater than the mussels' capacity for assimilation. The excess, if not too great, may pass unchanged through the digestive system but in case of a very great surplus most of the material is rejected by the palps and is discharged from the mantle cavity as pseudofeces. No satisfactory evidence of selection, except as to size, from among these small cells and particles has been obtained, although chemically injurious substances are rejected, together with the larger cells and other objects.

Digestion in the mussel, with the exception of starch and glycogen, appears to be mainly or wholly intracellular. Many of the smallest objects and particles are phagocytized by the cells lining the digestive diverticula. Others are ingested by phagocytic cells which migrate into the lumens of the stomach and intestine and later return with their ingested materials through the epithelial lining of the digestive tract and thence to the connective tissues of the body, as Yonge (1926, 1931) has so fully described for the oyster. Most of the local dinoflagellates and many of the diatoms are far too large to be assimilated in this manner. Some of the starch and glycogen, on the contrary, undergoes extracellular digestion in the stomach through the action of enzymes in the style. No evidence of the digestion of cellulose, which forms the covering walls of most neritic dinoflagellates, nor of any cells with completely closed cellulose walls, has been obtained (Fox and Coe, 1943).

Diatoms. These organisms, either living or dead, furnish a small portion of the mussels' nutrition. Their disintegration products are also utilized. They are usually present in numbers ranging from 1000 to 200,000 per liter but the large and spiny ones are not ingested. Many of those that enter the digestive tract are seized and digested by the phagocytic cells mentioned in a preceding paragraph, while others pass through the tract without apparent change.

The mean number of diatoms, as counted by the settling method, per liter of water for each month of the four years is shown in Figures 1 to 4 and the combined monthly averages for 1940, 1941 and 1943 in Figure 5. In none of these years has there been a direct correlation between the diatom populations and the mussels' growth rate, although positive correlations have been reported by Newcombe (1935) for Mya and by Nelson (1942) and others for oysters.

The number of diatoms in the water about the mussel beds has varied greatly from year to year. The average in 1940 was 38,700 per liter, in 1941 16,600, in 1942 12,600 and in 1943 54,300 (Figs. 1–4). The average monthly increase in the lengths of the mussels for the same years was 3.43 mm., 3.96 mm., 5.43 mm. and 5.10 mm., respectively. It is obvious that there was in these four years no direct correlation in the two groups of data. In spite of a four-fold increase in the number of diatoms in 1943 as compared with 1942 there was nevertheless a somewhat lower rate of growth in the mussels. This should not be surprising when it is realized that even if these organisms had been uniformly distributed in the water throughout the year, instead of occurring in dense swarms, the rate of filtration by the mussel is such that an adult animal could have secured no more than 200 million to 800 million per year. If all of these could have been fully utilized they would have furnished only a minute fraction of the material required for the upbuilding of the mussel's tissues and gametes. Such of these organisms as can be ingested by the mussel are so minute that it would require some 600 million to supply one gram of organic matter, while the adult mussel is estimated to need about 40 grams annually (Fox and Coe, 1943).

Bacteria. Bacteria are ingested in vast numbers (Figs. 1–3) but their total mass is so small that they have little quantitative influence on the mussels' nutrition (Fox and Coe, 1943).

Dinoflagellates. It has been mentioned that in each of the four years there was, generally, but with some conspicuous exceptions, a rather close correspondence between the monthly and yearly growth rates of the mussels and the abundance or scarcity of dinoflagellates in the water. In 1940 the average daily number of these organisms per liter of water was 12,100, as compared with 9880 in 1941, 54,750 in 1942 and 49,500 in 1943. The corresponding average monthly growth rates of the mussels were 3.43 mm. in 1940, 3.96 mm. in 1941, 5.43 in 1942 and 5.10 in 1943. From these figures alone it may be concluded that the mussels grow most rapidly in those years in which the populations of dinoflagellates are the largest.

More precise evidence as to this association however is furnished by an inspection of the monthly data as shown in Figures 1–4. It has been emphasized in a foregoing paragraph however that a large proportion of the living dinoflagellates, which may be ingested in vast numbers, usually pass apparently unchanged through the intestinal tract and often constitute much of the fecal material. Their cellulose walls cannot be digested by the secretions in the stomach or intestine and there is no satisfactory evidence that they are phagocytized by the cells of the digestive diverticula in any considerable numbers. Following the death of these unicellular organisms however, either before or after entering the mussel's digestive tract, they doubtless form particulate disintegration products which are readily assimilated. It is well known that species of Gonyaulax and less frequently of some other genera may be ingested in such numbers and the contained toxic substance accumulated in such quantity as to cause sickness or even death when the mussels are eaten by man.

It has been shown by Fox and Coe (1943) that the mussel filters the water at such a rate that the available supplies of dinoflagellates, even if they could be fully utilized, would furnish only a small fraction of the food which the mussels require for their growth and reproduction. Assuming a filtration rate of 2.5 liters per hour, or 22,000 liters per year, it would be necessary to have an average population of about 2000 of these cells per liter in order to supply one gram of organic matter in a year. This is only about two and one-half per cent of the amount which an adult mussel is estimated to require annually for the upbuilding of its tissues and gametes. During these four years the water has contained averages of 10,000 to 54,750 of these cells per liter but a large proportion of these were present in such dense swarms that relatively few of those that were drawn into the mantle cavities of the mussels could have been actually ingested; the others were presumably discharged as pseudofeces. Furthermore, as has been stated, many of those that are ingested usually pass through the digestive tract without visible change, while the large and spiny forms are seldom ingested.

Therefore any correlation between the abundance of dinoflagellates and the growth rates of the mussels must be merely indicative of other, associated sources of nutrition. The principal source is organic detritus.

Detritus. The organic detritus ingested by the mussel consists of various fragments of cells or of entire cells of minute size, as well as suspended proteins, lipids and polysaccharides. It may be recalled that the mussel obtains its food by secreting over its gills a thin sheet of mucus to which the particles are adsorbed. The mucus sheet with its attached particles is then drawn into the mouth. There is no evidence that the mussel is capable of securing substances in true solution until these have first been changed to particulate form through the agency of various unicellular organisms (Fox and Coe, 1943).

Consequently it may be concluded that, with the exception of refractory humus materials, cellulose, chitin and other indigestible substances, the total organic constituents of all marine organisms, from the smallest to the largest, are potential sources of nutrient for the mussel. After the death and disintegration of the animal or plant, the residual organic matter, or detritus, may remain suspended in the water for an indefinite period before it chances to enter the digestive system of the mussel. The amount available is obviously subject to great variation locally and it is to this variation that many of the differences in growth rates are ascribed.

Inspection of Figure 5, which indicates the combined average monthly growth rates for three years, will show that the rate increases from a low in January or February to a maximum in May, June and July. This period corresponds with the increasing reproduction of many of the invertebrates in the vicinity and elsewhere along the coast. The striped barnacle (*Balanus tintinnabulum*), for example, has minimum and maximum periods of reproduction coinciding almost precisely with the low and high growth rates of the mussels (Fig. 5). These reproductive periodicities are undoubtedly associated with similar variations in the detritus which the barnacles yield, since a large proportion of the free-swimming larvae die without finding a place of attachment and presumably less than one per cent of those which succeed in transforming to the adult stage survive to reach sexual maturity (Coe, 1932). The disintegrated bodies of those that perish doubtless supply more nutritive material to the mussel than can be obtained from the living phytoplankton.

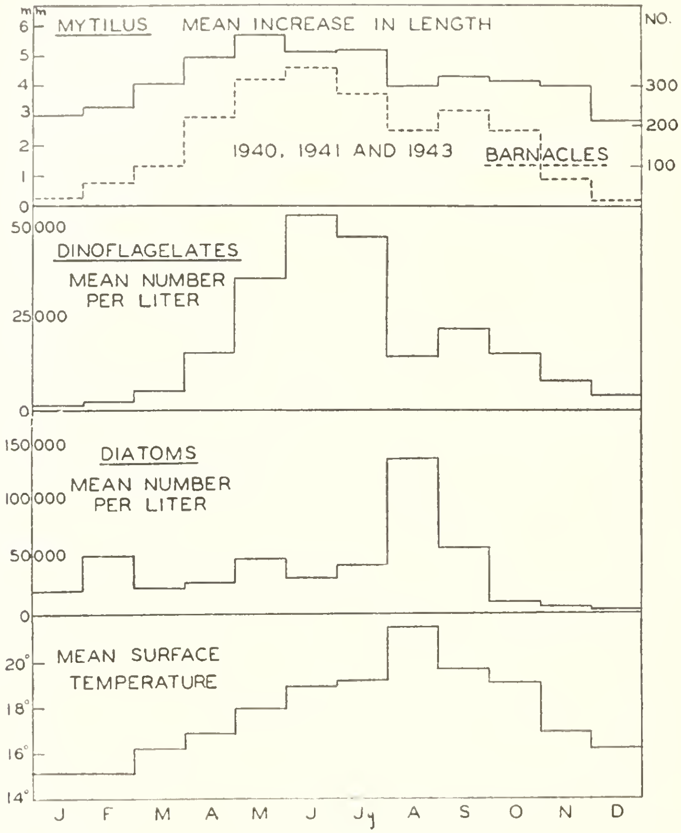

FIGURE 5. Correlations between the mean monthly increment in length of the experimental groups of mussels for the years 1940, 1941 and 1943, the mean numbers of dinoflagellates and diatoms per liter and the mean temperature of the water. The year 1942 is not included because of lack of complete data for the first five months of that year. Except for the decrease both in growth rate of the mussels and numbers of dinoflagellates during August, when the temperatures were highest, the general correspondence of three of these groups of data is evident. But this correspondence does not hold for the diatoms.

As indicative of the relative amount of organic detritus presumably available in each month, the estimated average numbers of barnacles which became attached to each square inch of surface of submerged plates in previous years have been included. These numbers indicate a close correlation with the growth rates of the mussels.

In some years the growth rate has been more or less distinctly bimodal, rising to a maximum in late spring or early summer, followed by an invariable decrease in

August and in three of the four years with another rise preceding the decrease at the end of the year. In three of the four years the dinoflagellates showed somewhat similar bimodal periodicities, with distinct spring and autumn maxima, and this has been shown to be the average condition of these organisms for the twenty preceding years (Allen, 1941). The diatom populations, on the contrary, reached a conspicuous maximum in August in 1940 and in 1943, although in many other years that has been a month of extremely low production.

The bimodal periodicities in the growth rates of the mussel are closely parallel with similar periodicities in the reproduction of many of the associated invertebrates, including the mussels themselves. The growth rates of the entire mussel population would obviously be complicated by the reproductive processes but these complications were avoided in this experiment by using sexually immature individuals.

It is unfortunate that no precise measurements are available relative to the local variations in the amount of organic detritus, but the seasonal differences in the rates of growth of the mussels and other detritus feeders presumably afford a fairly reliable criterion.

SUMMARY

This study offers additional evidence as to the complexity of the environmental conditions found along the shores of the ocean and which affect so profoundly the lives of the organisms residing there. Variations in the growth rates will obviously depend upon the interaction of several of these conditions, not the least important of which are the temperature and the character and abundance of the food supply.

Furthermore these environmental conditions are constantly changing, due in part to the continual motion of the water. At the locality where the foregoing observations were made, there are not only the variable currents caused by wind and tidal changes, but there is also a drift along the coast at a rate averaging four to five miles per day. Consequently the water in which the mussels are living and the conditions associated therewith may differ not only from month to month but also from day to day and even from hour to hour. In one week there may be ten to fifty times as much phytoplankton in the water as in the following week. The yearly averages are more stable but these may vary by more than five fold.

Monthly correlations, extending over four years, between the growth rates of the mussels and the prevalent environmental conditions offer conclusive evidence that the most rapid increase in size takes place at temperatures from 17 to 20° C., although growth continues less rapidly at 14° or lower. Feeding continues at a temperature as low as 7 to 8° and as high as 27 to 28°.

The average number of diatoms per liter was 38,700 in 1940, 16,600 in 1941, 12,600 in 1942 and 54,300 in 1943. The average number of dinoflagellates for the same years was 12,100, 9880, 54,750 and 49,500, respectively. The average monthly increment in the lengths of the mussels was 3.43 mm. in 1940, 3.96 in 1941, 5.43 mm. in 1942 and 5.11 mm. in 1943, indicating a yearly variation of more than 50 per cent.

It is obvious that the two years with the largest dinoflagellate populations have been conducive to the most rapid growth of the mussels, but an increase of four fold in abundance has been associated with an increased growth rate of only 42 per cent. The correlation noted is evidently not direct, since the living dinoflagellates

can supply only a small fraction of the mussels' nutritive requirements. Both mussels and dinoflagellates appear to thrive under the same environmental conditions. That the living diatoms and bacteria are of even less importance in the life of the mussel is indicated not only by the small amount of organic matter that they contain but also by the fact that the mussels grew most rapidly in the year with the smallest number of diatoms and least rapidly in the year when the number was three times as great.

More direct correlations with the growth rates of the mussels are found in the reproductive periodicities of various invertebrate populations which contribute so largely to the available organic detritus and thereby to the mussels' nutrition.

The principal food supply of this species of mussel consists of minute particles of organic detritus derived from the disintegration of the cells of all kinds of marine organisms, both animals and plants, supplemented by living and dead unicellular organisms of minute size as well as living and dead gametes. There is no evidence that organic matter in true solution can be utilized until after it has been changed into particulate form by the action of unicellular organisms.

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PHOTOCHEMICAL SPECTRAL ANALYSIS OF NEURAL TUBE FORMATION [1]

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INTRODUCTION

In recent years the analysis of morphogenesis has been concerned with the study of the chemical nature of substances which play a decisive role in developmental phenomena. In the field of neural induction the usual procedure used is that of extraction of the active material with solvents specific for a particular group of compounds; the degree of substitution is tested by implantation of the extracted material. This approach has failed to yield conclusive results because large quantities of tissue known to contain the normal inductor are not available. Investigators have been unable to determine whether induction resulting from implanted substances is the result of the direct action of the substance on the tissue or of a substance released in the reacting tissue. This technique is also subject to the criticism that two chemical substances may not necessarily be identical because they produce the same histological or morphological changes. It is a well-known fact that histological changes produced in the vagina and uterus by a number of artificial estrogens are identical with those produced by the natural estrogens (McKenzie, 1941).

The investigation of active substances need not be restricted to attempts to isolate them, although isolation and synthesis is the ultimate goal. If the action of a developmental substance is inhibited by a specific agent, a preliminary identification will have been made and it will be certain that the substance inactivated is operative in the organism. The use of chemical poisons has demonstrated the importance of this technique in the field of cellular oxidation. The technique of the photochemical inactivation of substances involved in the developmental processes has enabled the investigator to study the chemical nature of this material during its action in normal development.

The classical experiments of Warburg (1927) in the identification of the respiratory enzyme by absorption spectrophotometry show the importance of the technique of photochemical inactivation. This method involves the irradiation of a biological system with monochromatic radiation and the consequent inactivation of a chemical substance in the system. Absorption is measured indirectly in terms of a physiological or morphological change produced in the biological system. Warburg measured absorption by determining the change in oxygen consumption of yeast cells in the presence of carbon monoxide following irradiation with monochromatic light. In the present investigation absorption is measured in terms of the amount of energy required to inhibit the folding process in neural tube formation. By this method an indirect photochemical absorption spectrum of the material involved in a biological activity can be obtained. By comparing this spectrum with the absorption spectra of chemical compounds, information can be ascertained concerning the chemical nature of the material.

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[1] This paper is part of a dissertation presented to the faculty of the Graduate School of the University of Missouri in fulfillment of the requirement for the degree of Doctor of Philosophy.

A preliminary exploration showed that ultraviolet radiation would not inhibit the transformation of gastrula ectoderm into neural plate unless extremely large doses were used and the cells severely altered or killed. However, the folding process of neural tube formation was inhibited by very weak doses of ultraviolet light with little effect on the embryo in other ways. The neural plate merely continued to develop as a plate. The effect was uniform enough to compare quantitatively the effects of different wave-lengths. Consequently, an attempt was made to identify by its absorption spectrum a substance which is apparently of decisive importance in the process of neural tube formation.

Since most of the work concerned with neural tube formation has been done in Amphibia, it might be expected that this material would offer more advantages than any other. This is not the case. Amphibian embryos possess yolk granules and pigment which absorb and scatter incident radiation. Consequently, the photochemical efficiency curve obtained for inhibition of the folding process in Amphibia would not give a true measure of absorption. For this reason and because an abundant source of avian material was available, it was decided to use chick embryos in this investigation.

The author wishes to thank Dr. Daniel Mazia under whose direction the study was made. Grateful acknowledgment is made to Dr. F. M. Uber who permitted the author to make the thermopile measurements in his laboratory and to Dr. L. J. Stadler for use of the monochromator.

Materials and Methods

The material for this study consisted of the eggs of two breeds of the domestic fowl, the White Leghorn and the New Hampshire Red. In order to secure uniform results, all the eggs were obtained from two pens of hens, one of New Hampshire Reds and the other of White Leghorns. The University of Missouri poultry farm was the source of this material.

Each egg was incubated and the position of the blastoderm determined by candling. The egg was then placed in a Syracuse watch glass which contained modeling clay to hold the egg in place. Only sterile equipment was used in these experiments. The surface of the egg was sterilized with a piece of cotton which previously had been soaked in 70 per cent alcohol. An opening of 7 to 9 sq. mm. was cut in the egg shell by means of a small saw. This revealed the blastoderm through the shell membrane. After removal of the shell membrane with forceps, a sterile .9 per cent salt solution was used to float the embryo to the level of the surface of the egg shell. The age in terms of somites and general condition of each embryo was determined with a dissecting binocular; all embryos in which the neural tube was closed in any region and all abnormal embryos were discarded. The egg shell was marked with a pencil to indicate the position of the embryonic axis so that it could be placed parallel to the slit on the monochromator. The egg, which was tightly fixed in the watch glass by modeling clay, was placed upon a stand which had been attached to a rack and pinion. The egg was elevated until its surface was beneath a quartz prism which was situated at the slit on the monochromator. This "mechanical jack" enabled the investigator to place each embryo the same distance from the source of light. The experimental embryos were irradiated for varying lengths of time with monochromatic ultraviolet light. The control embryos were treated in exactly the same manner except that a glass microscopic slide was placed in the path of the light beam so that no ultraviolet light struck the embryo. Following irradiation, the opening in the egg shell was covered with a piece of a glass cover slip and sealed with a mixture of beeswax and paraffin. The eggs were then incubated for 30 hours or longer; the glass window was always placed down in order to prevent the blastoderm from adhering to the cover slip.

After incubation, the blastoderm was removed from the yolk by cutting around the periphery of it with iridectomy scissors and lifting it off with a metal spatula. The blastoderm was washed in saline and fixed with picro-sulfuric acid. Observations were made upon embryos in alcohol, from whole mounts, and from sectioned material. The whole mounts were stained with borax carmine and the sectioned material was stained with Delafield's hematoxylin or borax carmine.

In the majority of the experiments in this investigation, monochromatic radiation was obtained by means of a large crystal monochromator, described in detail by Uber and Jacobsohn (1938). The monochromator was operated in a horizontal position. Since it was necessary to obtain a vertical beam of light in order to irradiate the embryo, a small quartz prism was placed at the slit on the monochromator. The source of light for the monochromator was a vertical mercury arc which operated at 4 amperes on a 110 volt direct current.

In order to determine the incident dose in ergs/mm.^{2} on the embryos, the monochromatic source was calibrated with a surface-type vacuum thermopile. The thermopile had been calibrated previously with a standard carbon-filament lamp (C–241) obtained from the United States Bureau of Standards. The dosage in ergs/mm.^{2} emitted by the monochromatic source was determined by comparing the deflection which it produced with that produced by the standard lamp.

In the other experiments a mercury discharge tube served as a source of radiation. It was of the Hanovia Sc–2537 type operating at 120 milliamperes and 5000 volts. The transformer was a Jefferson luminous type. Since spectral studies show that such discharge tubes frequently have an additional line around 1800 Å (Landen, 1940), a water filter was placed in the path of the beam in order to absorb the radiation of the shorter wave length. A slit of approximately the same size as that of the monochromator used in this investigation was made on the bottom of the filter in order to approximate the experimental set-up with the monochromator.

OBSERVATIONS

Histological studies of irradiated and control embryos

Chick embryos ranging in age from the primitive streak to the 8-somite stage were irradiated with monochromatic ultraviolet light of wave lengths 2483, 2537, 2576, 2650, 2699, 2804, 2894, 2967, and 3130 Å and subsequently incubated for a period of approximately 30 hours.

The smallest doses produce no detectable changes; the first visible effects to appear as the dose is increased are on the formation of the neural tube. The neural folds fail to close and instead form flat or half-folded neural plates. In some embryos this occurs in the anterior part of the body; however, in other cases, it is present only in the middle portion. Large doses result in destruction of cells and death of the embryo.

In the consideration of the effect of monochromatic radiation on a developmental process one of the first questions to arise is this: Is it possible to set up a quantitative standard of measurement for comparing the effectiveness of different wave lengths? Such a standard would be a morphological *endpoint*. The procedure would be to compare doses required to attain such an endpoint. If the data are significant, the results should be the same regardless of the particular endpoint chosen. In the present study two morphological endpoints are used: (1) failure of the neural tube to close for a distance of one-third its length in 50 per cent of the cases and (2) failure of the neural tube to close for a distance of one-half its length in 50 per cent of the cases. This investigation is concerned in particular with the embryos irradiated with the amounts of energy necessary to produce these two morphological endpoints. The description is made from a study of embryos in 70 per cent alcohol, sectioned embryos, and whole mounts.

The primary effect of radiation is on the neural plate. Embryos irradiated with wave lengths 2483, 2537, 2576, 2650, 2699, 2804, and 2894 Å are very uniform in appearance. A broad flat plate is present in the anterior one-third to five-sixths of the embryo; the neural tube is nearly always closed in the posterior end. The anterior end of the neural plate bends around the anterior tip of the free head and extends to the ventral surface. The optic cups and infundibulum develop from the portion of the neural plate on the ventral surface of the free head. A lens forms in most cases. A typical case with a broad flat plate, optic cups, lenses, and infundibulum is shown in a section through the anterior end of an embryo irradiated with wave length 2804 Å (Plate I, Figure 1). A section at a more posterior level is shown in Plate I, Figure 2; this embryo was irradiated with wave length 2537 Å. Observations show that the auditory pits are normal in appearance in every case. A group of cells which is probably the neural crest often lies adjacent to the lateral edges of the neural plate. In the region of the rhombencephalon the motor roots of the spinal nerves are present. In the posterior end of the embryo a double neural tube occurs occasionally.

The broad flat plate which is present in embryos of this group is nearly uniform in thickness except in the region of the midline. In this region the plate is thinner (Plate I, Figures 1 and 2). The volume of the broad plate is much larger than that of the neural plate of a 6-somite embryo. Mitoses are abundant on the upper surface of the plate and on the inside portion of the closed region of the neural tube. In a few embryos a small group of cells is present on the surface at the lateral edges of the neural plate. They are filled with granules and irregular in shape. In these cases the lateral ectoderm is similar in appearance to these cells; otherwise, the lateral ectoderm appears normal. In some cases it continues to grow and expand so that a projecting group of cells forms on the dorsal surface of the neural plate.

Another group of embryos is characterized by the presence of a flat or folded neural plate in the middle portion of the body. A complete neural tube forms in the anterior part of the embryo, but it is abnormal in shape and smaller than a

PLATE I

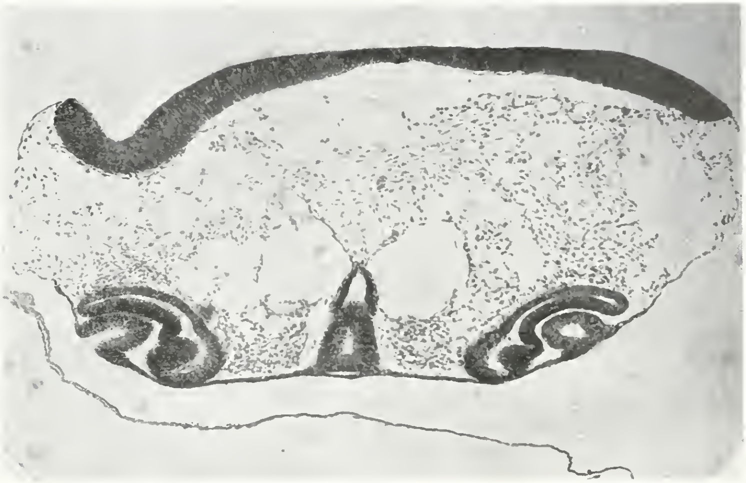

FIGURE 1. Anterior end of an embryo irradiated with approximately 71 ergs/mm.^{2} at wave length 2804 Å and subsequently incubated for 30 hours, showing the broad flat neural plate, optic cups, lens, infundibulum, gut, dorsal aortae, small notochord, and disorganized mesenchyme beneath the neural plate. × 107.

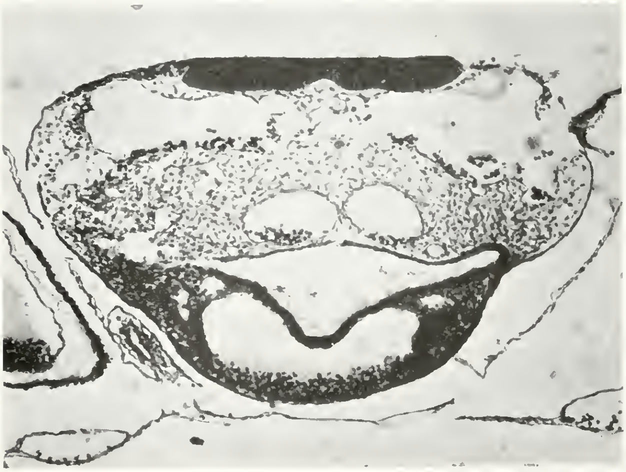

FIGURE 2. At the level of the heart of an embryo irradiated with approximately 95 ergs/mm.^{2} at wave length 2537 Å and subsequently incubated for 30 hours, showing the flat neural plate which possesses a well-defined floor plate, small notochord, dorsal and ventral aortae, heart, and gut. × 107.

normal tube. The typical thin roof plate of the myelencephalon fails to develop. The auditory pits and optic cups are normal.

The cellular appearance of the notochord in the irradiated embryos is the same as that of a normal embryo. Measurements of the cross-section area of the notochord show that it varies greatly at different anterior-posterior levels. The notochord is invariably separated from the neural plate; in the region of the neural tube the notochord lies in contact with the floor plate. In Plate I, Figures 1 and 2 illustrate the small size and relative position of the notochord. As far as can be detected, the somites are normal. In many cases, the mesenchyme beneath the neural plate is abnormally vesiculated in places and considerably disorganized (Plate I, Figure 1). Stained sections show that the mesenchyme beneath the neural plate has been injured; this is suggested by the dark appearance of its cells. The vascular system is well developed; however, the size of the vascular bed is smaller than that of control embryos.

The embryos which were irradiated with wave length 2967 Å are different in appearance from those described previously. The explanation for this is that extremely large doses had to be used in order to produce a detectable effect. In most cases the neural tube forms only in the most anterior part of the embryo. It is very small and abnormally shaped, being extremely flattened dorsoventrally. Occasionally the tube fails to develop and a neural plate is present in the anterior region. In the posterior three-fourths to four-fifths of the body the neural plate is either a disorganized mass of cells or completely absent. The superficial ectoderm appears normal and forms a continuous layer of flat epithelial cells dorsal to the neural plate.

In the embryos irradiated with wave length 2967 Å, the cellular structure of the notochord is normal in appearance. However, the notochord is not in contact with either the neural tube or plate in most regions. The somites are either highly disorganized or absent. The vascular system is poorly developed.

Observations of 111 control embryos which were made from embryos in 70 per cent alcohol, whole mounts, and sections show that they are normal in 100 per cent of the cases. It will be remembered that all embryos were examined immediately before irradiation and the abnormal ones discarded. This explains the fact that all control embryos are normal.

From this description, it is evident that all wave lengths except 2967 Å produce a uniform effect on neural tube formation; consequently, quantitative studies of the relative efficiency of different wave lengths in preventing closure of the neural tube can be made.

The effect of radiation on mitosis and volume of the central nervous system

The purpose of this study is to determine if radiation has a detectable influence on mitosis and volume changes with the low doses used. Mitotic counts and measurements of the area of cross sections of the central nervous system were made on the embryos which were described histologically in the previous section. An analysis of the effect of radiation on mitosis was made by counting the number of mitoses in three sections of the neural plate or tube at each of three different levels. Volume was measured indirectly by determining the cross-section area of one of

TABLE I

Mitotic counts and cross-section measurements of irradiated and control embryos

Group of embryos	Number of mitoses per section per embryo	Cross-section area per embryo	Mitoses per cross-section area unit
Level *a*			
Embryos irradiated at wave lengths from 2483 to 2804 Å (10)*	45.90±14.7	1189.2±278	.038
Embryos irradiated at wave lengths 2894 and 2967 Å (4)	20.75±9.0	719.5±95	.029
Control embryos (5)	44.80±15.0	1504.6±409	.030
Level *b*			
Embryos irradiated at wave lengths from 2483 to 2804 Å (10)	7.80±2.6	251.3±34	.031
Embryos irradiated at wave lengths 2894 and 2967 Å (4)	1.75±2.1	93.75±47	.018
Control embryos (5)	12.60±4.7	379±115	.033
Level *c*			
Embryos irradiated at wave lengths from 2483 to 2804 Å (10)	6.90±2.1	191.5±51	.036
Embryos irradiated at wave lengths 2894 and 2967 Å (4)	4.75±5.6	99.5±85	.048
Control embryos (5)	10.00±2.0	284.60±46	.035

Level *a* consists of three sections adjacent to the anterior end of the notochord; level *b* is represented by the middle section of the central nervous system and this level is determined by counting the total number of sections of the central nervous system; and level *c* is ten sections posterior to the most posterior section in which the neural tube has failed to close in the irradiated embryos and a comparable section in control embryos. In the irradiated embryos in which the counts were made, level *c* is always in the posterior quarter of the nervous system. In determining the number of mitoses per section per embryo, counts were made on three sections at each level of each embryo. The number of mitoses per section at each level was obtained by averaging these three figures; the number of mitoses per section per embryo was determined by averaging the number of mitoses per section for the group of embryos. Volume was measured by determining the cross-section area of one of the sections at each level in which the mitoses were counted; cross-section area per embryo was obtained by averaging the cross-section area for the group of embryos. The mean deviation has been calculated for the average values of the number of mitoses and cross-section area.

* The figures in parenthesis represent the total number of embryos studied in each group.

the sections at each level in which the mitoses were counted. This was accomplished by tracing the outline of the neural plate or tube on millimeter paper by means of a camera lucida and then counting the number of square millimeters within the outline. No attempt was made to transfer the values obtained into absolute ones, since this investigation is concerned only with relative data.

TABLE II

The effect of an increased incubation period on closure of the neural tube

Egg number	Irradiated with 143.6 ergs/mm.²				Irradiated with 170.0 ergs/mm.²			
	30-hour incubation		54-hour incubation		30-hour incubation		54-hour incubation	
	Age in somites at time of irradiation	Distance open	Age in somites at time of irradiation	Distance open	Age in somites at time of irradiation	Distance open	Age in somites at time of irradiation	Distance open
1	6	1/3	6	1/3	6	1/2	6	0
2	6	1/4	4	1/3	3	1/2	5	1/5
3	6	3/4	6	0	2	1/2	6	0
4	6	0	5	0	6	1/5	6	1/4
5	6	open	6	0	6	1/2	6	0
6	6	1/3	1	0	0	0	6	0
7	6	1/3	7	0	7	1/4	3	1/4
8	5	0	4	0	7	3/4	4	1/3
9	6	open	4	0	6	2/3	7	1/5
10	3	open	6	0	6	1/3	6	open
11	2	3/4	6	0	8	1/4	8	1/4
12	3	1/3	6	0	2	0	3	0
13	0*	0	6	0	3	3/4	5	1/2
14	3	0	4	1/3	2	1/2	7	0
15	6	1/2	6	1/3	2	3/4	6	1/2
16	0	0	6	0	3	1/2	7	0
17	6	1/3	5	1/4	7	1/3	6	0
18			6	0			7	0
19			6	0			7	1/3
20							6	1/3
21							6	0
22							8	0
23							4	0
24							6	1/3
25							7	1/6
% open 1/3 way or more		47		21		70		26

* Embryos designated as having no somites were in the primitive streak stage.

The results are shown in Table I. From the data presented, it is concluded that the results with wave lengths 2483 to 2804 Å are not decisive enough to establish an influence of radiation on mitosis and volume. However, wave lengths 2894 and 2967 Å are very effective in decreasing mitosis and volume.

The effect of an increased incubation period on closure of the neural tube

The purpose of this experiment is to determine whether or not the neural folds which have failed to close in irradiated embryos will close if the incubation period is increased. Embryos ranging in age from the primitive streak to the 8-somite stage were irradiated with the mercury discharge tube with doses of 143.6 and 170.0 ergs/mm.^{2}. The embryos were then incubated for periods of 30 and 54 hours. After removal and fixation of the embryos, observations were made with a dissecting binocular. The results are shown in Table II. Columns 3, 5, 7, and 9 which are designated as "Distance open" refer to the distance for which the neural tube has failed to close. The per cent of embryos in which the neural tube is open for a distance of one-third its length or more decreases with an increase in the period of incubation. For the 143.6 ergs/mm.^{2} group the per cent decreases from 47 to 21; in the 170.0 ergs/mm.^{2} group the per cent decreases from 70 to 26. This experiment shows that an increased incubation period results in partial closure of the region of the central nervous system which had failed to close after 30 hours incubation.

Method of calculation of the incident energy on the embryos

In the histological study of the irradiated embryos described in the first section of the observations, all wave lengths except 2967 Å are found to produce the same qualitative effects. Since the effect of radiation is to inhibit the folding process, a quantitative comparison of the relative photochemical efficiency of different wave lengths can be made. The amount of incident energy in ergs/mm.^{2} required to inhibit folding was determined for the wave lengths used in this investigation. To facilitate a comparison of the relative efficiency of these wave lengths, two morphological endpoints, namely, the inhibition of closure of the neural tube for a distance of one-third and for a distance of one-half its length in 50 per cent of the embryos were chosen. *Attention is called to the fact that these morphological endpoints have dimensions of energy and for this reason are an indirect measure of absorption.* In order to determine the incident energy on the eggs, the intensity of the

TABLE III

Absolute intensity of monochromatic radiation

Wave length	Cm. deflection	Ergs/mm.^{2}/sec. producing 1 cm. deflection	Ergs/mm.^{2}/sec.
2483	1.5	.726	1.089
2537	5.2	.726	3.775
2576	.6	.726	.436
2650	3.8	.726	2.759
2699	.9	.726	.653
2804	2.1	.726	1.525
2894	1.1	.726	1.799
2967	2.9	.726	2.105
3130	12.5	.726	9.075

radiation at the egg surface for each wave length was measured by means of a surface-type vacuum thermopile. The thermopile measurements are shown in Table III, where "cm. deflection" refers to the number of centimeters the galvanometer needle was deflected when the intensity of each wave length was measured. By multiplying this value by .726 ergs/mm.^{2}/sec., which is the amount of energy producing a deflection of 1 cm., the number of ergs/mm.^{2}/sec. emitted by each line was obtained.

The total numbers of embryos irradiated at wave lengths 2483, 2537, 2576, 2650, 2699, 2804, 2894, 2967, and 3130 Å are 75, 93, 45, 100, 68, 79, 42, 54, and 16, respectively. The results obtained with incident doses on the egg stronger or weaker than the doses required to inhibit closure of the neural tube for distances of one-third and one-half its length in 50 per cent of the cases are summarized in Table IV.

Table IV

Results with experimental doses stronger and weaker than the endpoint dose

Wave length	Incident dose on egg in ergs/mm.^{2}	Open $\frac{1}{3}$ way or more	Open less than $\frac{1}{3}$ way or closed	Per cent open $\frac{1}{3}$ way or more	Open $\frac{1}{2}$ way or more	Open less than $\frac{1}{2}$ way or closed	Per cent open $\frac{1}{2}$ way or more
2483	196.06	16	25	39	13	28	32
	261.36	10	9	53	6	13	32
2537	113.25	4	14	22			
	169.89	19	9	68	11	17	39
	226.50				9	6	60
2576	130.80	10	12	45	5	17	23
	156.96	13	5	72	11	7	61
2650	207.00	14	16	47	6	24	20
	248.40	14	9	60	12	11	52
2699	195.90	4	6	40			
	215.49	2	2	50			
	235.08	11	10	52	10	11	48
	274.26				4	1	80
2804	137.25	17	17	50	15	19	44
	183.00				4	1	80
2894	239.70	6	10	38	5	11	31
	287.64	12	6	67	8	10	44
2967	631.50	0	13	?	0	13	?
	757.80	0	17	?	0	17	?
3130	5445.00	0	7	?	0	7	?
	9256.50	0	1	?	0	1	?

The figures in columns 3, 4, 6, and 7 represent the number of embryos irradiated; the figures in columns 5 and 8 refer to the per cent of embryos irradiated. "Open one-third way" or "open one-half way" means that the neural tube is open at least one-third or one-half its length; "open" refers to cases in which the neural tube is open less than one-third or one-half way; and "closed" indicates that the neural tube is closed.

In order to determine the amount of energy incident on the embryo, it is necessary to measure the amount of light transmitted by the vitelline membrane. The ultraviolet transmission of the vitelline membrane was measured by Uber, Hayashi, and Ells (1941). These measurements were made with a Spekker photometer and a Hilger medium quartz spectrograph. Three vitelline membranes were studied and the results are shown in numerical form in Table V for each wave

TABLE V

Ultraviolet transmission of three vitelline membranes
(Hilger spectrograph dàta)

Wave length	Per cent transmission			
	Membrane 1	Membrane 2	Membrane 3	Average
2483	6.35	4.65	3.05	4.68
2537	8.00	6.20	4.20	6.13
2576	9.90	7.00	5.20	7.37
2650	10.00	7.80	6.80	8.20
2699	10.00	8.00	7.45	8.48
2804	11.00	8.10	8.00	9.03
2894	16.30	13.65	11.00	13.65
2967	21.80	20.70	15.85	19.45
3130	26.10	30.10	30.10	28.76

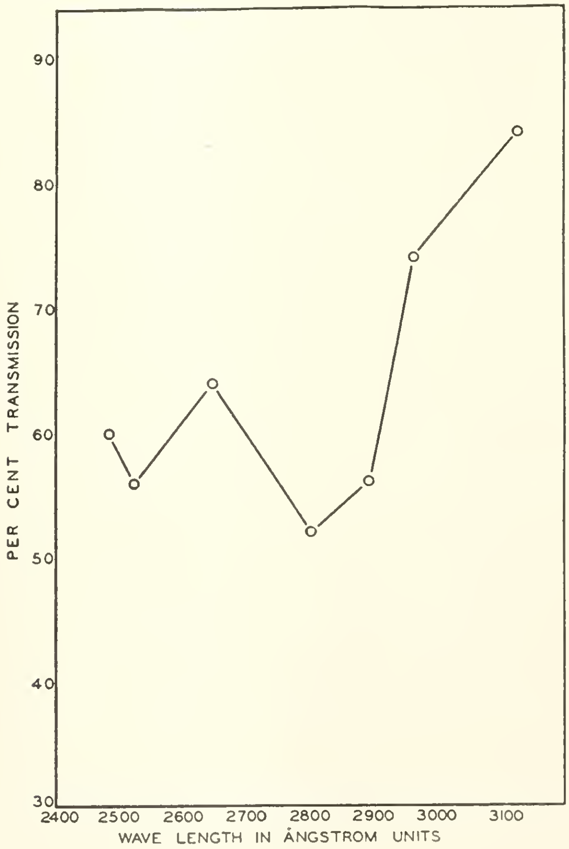

FIGURE 1. Ultraviolet transmission by vitelline membrane (T. Hayashi, unpublished).

length used in this study. In addition to the spectrograph data, Mr. Teru Hayashi (unpublished) measured the ultraviolet transmission of the vitelline membrane with a photocell and a quartz microscope. His data are shown in Figure 1. The amount of energy incident on the embryo was then calculated for these two sets of data on transmission by the vitelline membrane by multiplying the per cent transmission by the vitelline membrane by the incident energy on the egg.

Relative photochemical efficiency curves

The results of the calculations of the incident energy on the embryos are presented in Tables VI and VII in which correction for absorption by the vitelline membrane is made with the Hilger spectrograph; in Tables VIII and IX, the photocell and quartz microscope transmission measurements are used for this correction. At most wave lengths the doses recorded in the tables are just stronger or weaker than the endpoint doses, namely, the amounts of energy necessary to prevent closure of the neural tube for distances of one-third and one-half its length in 50 per cent of the embryos. At wave length 2804 Å, a dose was used which prevented neural tube formation in exactly 50 per cent of the cases when one-third the length of the tube was used as an endpoint. When the doses used did not produce the endpoint, the endpoint dose was determined by interpolation. The validity of this interpolation is based upon the assumption that the effect produced is directly proportional to dose. This assumption was used during the course of this investigation to predict the dose which would produce the endpoint. These predictions were fairly accurate, particularly in view of the small number of em-

TABLE VI

Incident energy on embryos for inhibition of closure of ⅓ the length of the neural tube

(Hilger spectrograph data)

Wave length	Incident dose on egg in ergs/mm.^{2}	Per cent transmission by vitelline membrane	Per cent open ⅓ way	Incident dose on embryo in ergs/mm.^{2}	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	4.68	39	9.18	.109	11.58	.086
	261.36		53	12.23	.082		
2537	113.25	6.13	22	6.84	.146	9.01	.110
	169.89		68	10.41	.096		
2576	130.80	7.37	45	9.64	.104	10.00	.100
	156.96		72	11.57	.086		
2650	207.00	8.20	47	16.97	.059	17.75	.056
	248.40		60	20.37	.049		
2699	195.90	8.48	40	16.61			
	215.49		50	18.27	.055	18.27	.055
	235.08		52	19.93			
2804	137.25	9.03	50	12.39	.080	12.39	.080
2894	239.70	13.65	38	32.62	.031	35.37	.028
	287.64		67	39.26	.025		
2967	631.50	19.45	?	122.83	.0081	?	?
	757.80		?	147.39	.0068	?	?
3130	5445.00	28.76	?	1565.98	.0006	?	?
	9256.50		?	2662.17	.0004	?	?

TABLE VII

Incident energy on embryos for inhibition of closure of ½ the length of the neural tube
(Hilger spectrograph data)

Wave length	Incident dose on egg in ergs/mm.^{2}	Per cent transmission by vitelline membrane	Per cent open ½ way	Incident dose on embryo in ergs/mm.^{2}	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	4.68	32	9.18	.109	?	?
	261.36		32	12.23	.082		
2537	169.89	6.13	39	10.41	.096	12.12	.082
	226.50		60	13.88	.072		
2576	130.80	7.37	23	9.64	.104	11.01	.090
	156.96		61	11.57	.086		
2650	207.00	8.20	20	16.97	.059	20.16	.050
	248.40		52	20.37	.049		
2699	235.08	8.48	48	19.93	.050	20.20	.050
	274.26		80	23.25	.043		
2804	137.25	9.03	44	12.39	.081	13.08	.076
	183.00		80	16.52	.061		
2894	239.70	13.65	31	32.62	.031	42.32	.023
	287.64		44	39.26	.025		
2967	631.50	19.45	?	122.83	.0081	?	?
	757.80		?	147.39	.0068		
3130	5445.00	28.76	?	1565.98	.0006	?	?
	9256.50		?	2662.17	.0004		

TABLE VIII

Incident energy on embryos for inhibition of closure of ⅓ the length of the neural tube
(Quartz microscope and photocell data)

Wave length	Incident dose on egg in ergs/mm.^{2}	Per cent transmission by vitelline membrane	Per cent open ⅓ way	Incident energy on embryo in ergs/mm.^{2}	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	60	39	117.63	.0085	148.42	.0067
	261.36		53	156.82	.0064		
2537	113.25	56	22	63.42	.0158	82.75	.0121
	169.89		68	95.14	.0105		
2576	130.80	58.5	45	76.52	.0131	79.35	.0126
	156.96		72	91.82	.0109		
2650	207.00	64	47	132.48	.0075	137.78	.0073
	248.40		60	158.98	.0063		
2699	195.90	59	40	115.58	.0087		
	215.49		50	127.14	.0080	124.14	.0080
	235.08		52	138.69	.0073		
2804	137.25	52	50	71.37	.0140	71.37	.0140
2894	239.70	56	38	134.20	.0074	145.32	.0069
	287.64		67	161.08	.0062		
2967	631.50	74	?	467.31	.0021	?	?
	757.80			560.77	.0018		
3130	5445.00	84	?	4573.80	.00022	?	?
	9256.50		?	7775.46	.00013		

bryos obtained at stronger and weaker doses which served as a basis for the predictions. In most cases three or four experiments were run and comparable results were obtained in each experiment. Furthermore, if the experimental data which most closely correspond to the endpoint are used instead of the interpolated data, the maxima and minima of the curves are not significantly changed.

Although the embryos at the time of irradiation varied in age from the primitive streak to the eight somite stage, the results were not altered. Davis (1942) recorded the age of each embryo at the time of irradiation and the results show that the effect of radiation is independent of age for the small age range used in this group of experiments. Furthermore, it was possible to predict the experimental results that would be obtained with slightly larger or smaller doses regardless of the age of the embryos used.

Table IX

Incident energy on embryos for inhibition of closure of ½ the length of the neural tube

(Quartz microscope and photocell data)

Wave length	Incident dose on egg in ergs/mm.^{2}	Per cent transmission by vitelline membrane	Per cent open ½ way	Incident energy on embryo in ergs/mm.^{2}	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	60	32	117.63	.0085	?	?
	261.36		32	156.82	.0064		
2537	169.89	56	39	95.14	.0105	111.74	.0089
	226.50		60	126.84	.0079		
2576	130.80	58.5	23	76.52	.0131	87.39	.0114
	156.96		61	91.82	.0109		
2650	207.00	64	20	132.48	.0075	157.32	.0064
	248.40		52	158.98	.0063		
2699	235.08	59	48	138.69	.0073	153.22	.0065
	274.26		80	161.81	.0062		
2804	137.25	52	44	71.37	.0140	75.34	.0133
	183.00		80	95.16	.0105		
2894	239.70	56	31	134.2	.0074	172.71	.0058
	287.64		44	161.08	.0062		
2967	631.50	74	?	467.31	.0021	?	?
	757.80			560.77	.0018	?	?
3130	5445.00	84	?	4573.80	.00023	?	?
	9256.50		?	7775.46	.00013		

Interpolations cannot be made at wave lengths 2967 and 3130 Å. A large percentage of the tubes are open in the embryos which were irradiated with wave length 2967 Å; however, none are open as much as one-third the length of the neural tube. Embryos irradiated with 495.20 ergs/mm.^{2} or less show a slight injury, while the stronger doses used produce considerable injury. In view of this and since 757.80 ergs/mm.^{2} result in failure of the neural tube to close in 35 per cent of the cases, a dose of approximately 757.80 ergs/mm.^{2} is chosen as an endpoint dose. Wave length 3130 Å is found to be ineffective since a dose of 5445.00 ergs/mm.^{2} fails to produce a detectable change in the embryos.

The reciprocal of the calculated endpoint dose in ergs/mm.^{2} is plotted against wave length for each of the four sets of data and the photochemical efficiency curves are presented in Figures 2, 3, 4, and 5. All four curves show two well-defined absorption maxima at wave lengths 2576 and 2804 Å and a minimum at wave lengths 2650–2700 Å. The validity of using a morphological endpoint as a measure of the effect produced is shown by the fact that the curves for the two different morphological endpoints are almost identical in shape. Furthermore, the curves are very similar in shape regardless of the data used in correction for absorption by the vitelline membrane. This shows that the differences in effectiveness of different wave lengths as shown in the photochemical efficiency curves are truly significant.

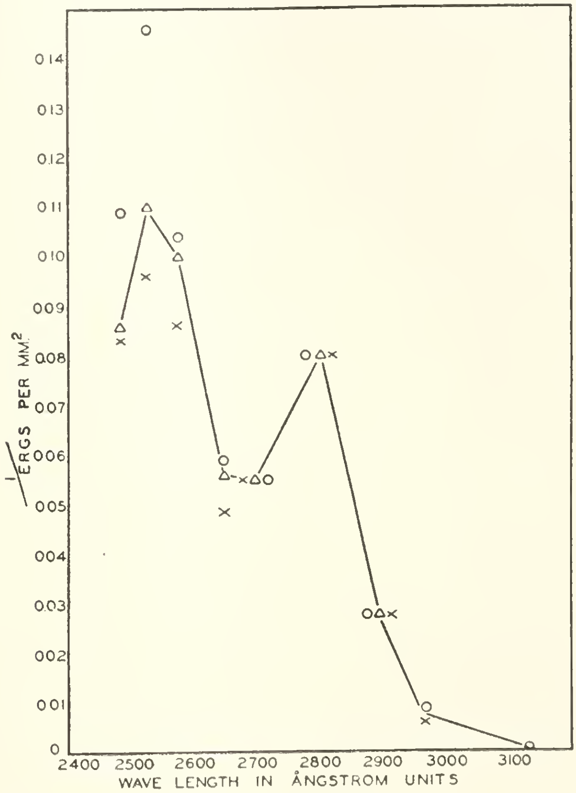

Figure 2. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-third its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with Hilger spectrograph. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

Discussion

Histological studies were made in order to determine whether a comparable unit of ultraviolet effect on the folding process was obtained at all effective wave lengths. Observations of the embryos irradiated with the amounts of energy necessary to produce the two morphological endpoints, namely, failure of the neural tube to close for distances of one-third and one-half its length in 50 per cent of the cases, show that the most apparent effect of ultraviolet light is on the neural plate. Instead of a neural tube forming, a broad flat plate develops. In a few cases the lateral ectoderm appears to be injured as suggested by the dark granular appearance of its cells. The groups of cells observed on the surface of the neural plate are probably derived from the lateral ectoderm since the two are very similar in appearance. If this is true, then the isolation of these groups of cells likewise indicates that the lateral ectoderm is injured. However, the magnitude of this effect is small; otherwise, the lens and otic vesicle would not develop in an apparently normal manner. Consequently, the effect of radiation on the ectoderm is not nearly as great on the lateral ectoderm as it is on the neural plate.

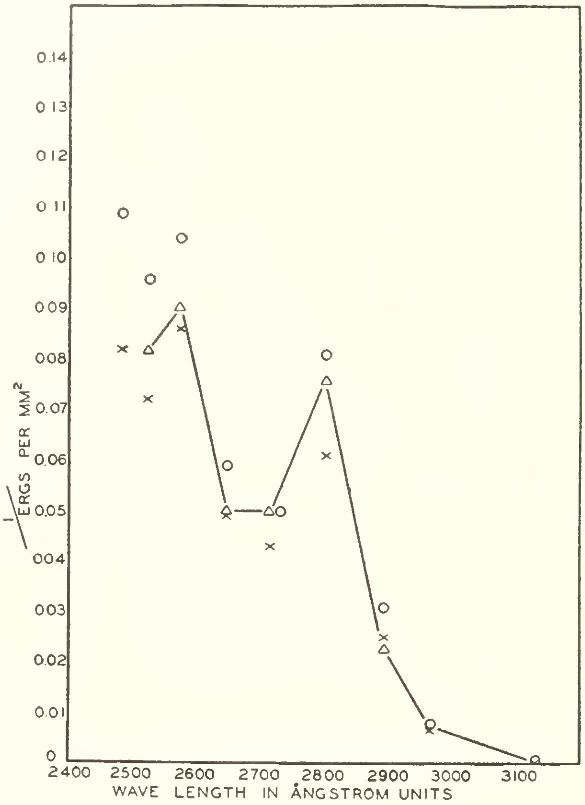

Figure 3. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-half its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with Hilger spectrograph. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

Only mesodermal derivatives which lie directly beneath the ectoderm are injured. Although no detectable change in the cellular appearance of the notochord was observed, the cross-section measurements indicate that the notochord is affected. The mesenchyme beneath the ectoderm is also injured. This interpretation is made from the dark and disorganized appearance of the mesenchyme cells. The somites are normal in appearance. It should be emphasized that the effects of radiation on the ectoderm and mesoderm discussed previously are uniform for all wave lengths except 2967 Å.

As previously stated, the most apparent effect of ultraviolet light is to prevent closure of the neural tube. Furthermore, failure of the neural plate to form a neural tube seems to be restricted to an effect upon the folding process because radiation does not appear to produce other effects on the neural plate cells. The results presented in Table II show that prolonged incubation leads to closure of the neural tube in regions which were open after 30 hours incubation. This probably can best be interpreted as indicating that material and not the capacity to produce or utilize material is altered. Microscopic observations fail to reveal any abnormal changes in the cells of the neural tube. The volume of the neural plate or

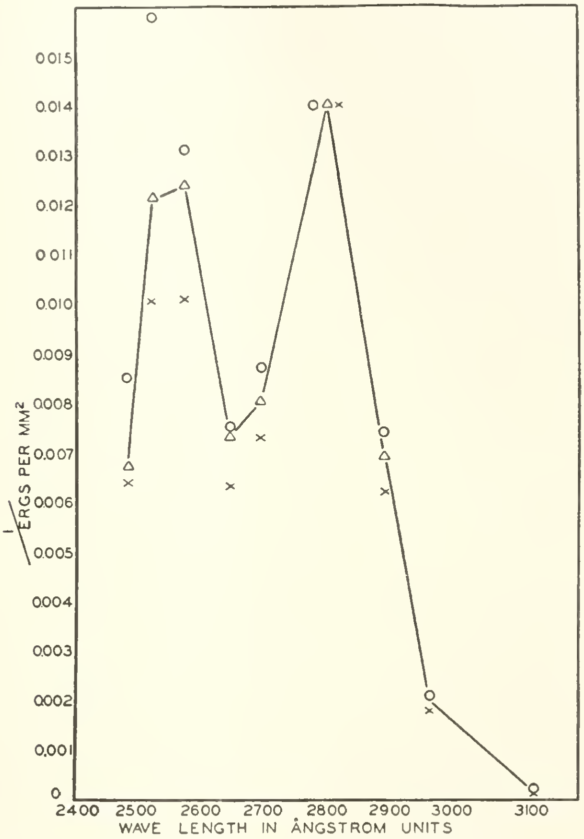

FIGURE 4. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-third its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with the photocell and quartz microscope. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

tube has increased considerably. In certain regions, the motor roots of the spinal nerves grow out of the neural plate which shows that histological differentiation continues even though folding of the plate as a whole has been inhibited.

Mitoses are very abundant and normal in position. The quantitative determinations presented in Table I suggest that mitosis is slightly affected by radiation at wave lengths from 2483 to 2804 Å. However, enough data have not been analyzed to settle this question. In view of the evidence presented by others on the effect of radiation on avian material, it seems unlikely that radiation has an effect at these small doses. Mayer and Schreiber (1934) found that doses of radiation several times as large as those used in this investigation were required to inhibit cell division of chick fibroblasts and chondroblasts in tissue culture. Even if mitosis is affected, the energy involved in producing this effect is evidently not involved in preventing folding. This reasoning is based upon the observation that

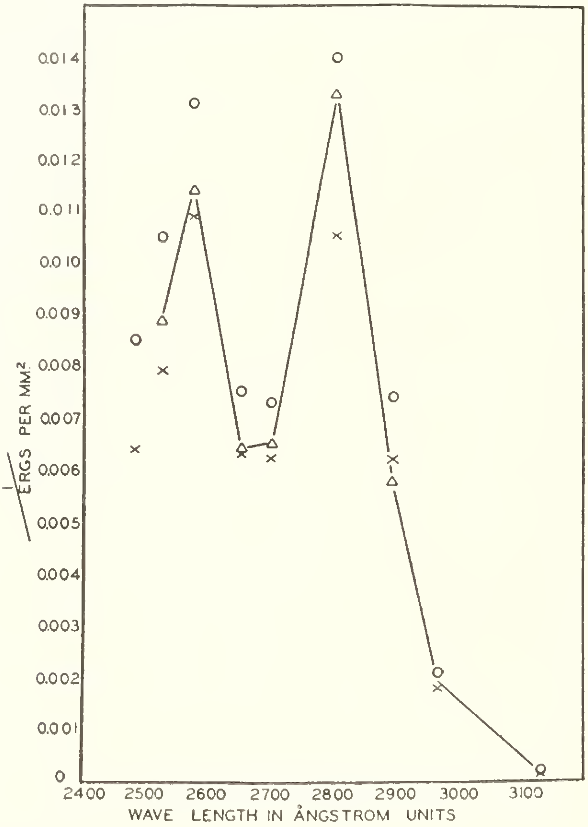

FIGURE 5. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-half its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with the photocell and quartz microscope. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

doses at wave lengths 2894 and 2967 Å which affect mitosis do not influence folding. The same inverse relation is found in the case of the cross-section measurements. Consequently, the data which have been analyzed indicate that a decrease in mitosis and volume is not causally related to inhibition of the folding process. This suggests that the incident radiation which is required to prevent folding is not involved in nuclear or volume changes and, consequently, is not absorbed by the cellular material engaged in these changes. From the discussion of the histological observations, mitotic counts, and cross-section measurements, it is evident that a comparable unit of ultraviolet effect on inhibition of the folding process is obtained at different effective wave lengths. In view of this, it is possible to obtain a photochemical efficiency curve for this process.

In order to determine accurately the photochemical efficiency of different wave lengths, corrections have been made for the energy absorbed by the material which screens the embryo. The two possible sources of error are the albumin and the vitelline membrane. The albumin present above the embryo after a 24 hour incubation period is negligible. It is unlikely that any albumin present is absorbing incident radiation since wave length 2804 Å which is most effective in inhibiting folding is most strongly absorbed by albumin.

On the other hand, the vitelline membrane absorbs a large per cent of the incident light. As can be seen by an examination of the data for the transmission measurements, different results were obtained by the two methods. With the spectrograph method only the amount of light transmitted by the vitelline membrane is measured and the values obtained by calculation of the incident energy on the embryo are minimum. The photocell and quartz microscope measure not only transmitted but some scattered radiation. Since the vitelline membrane lies in contact with the surface of the embryo, most of the scattered radiation is likely absorbed. In view of this the photocell measurements give a better insight into the actual amount of energy incident on the embryo. It should be pointed out, however, that the exact amount of scattered radiation measured with the photocell and quartz microscope is dependent upon certain experimental conditions such as the distance of the object from the objective and the diameter of the opening in the iris diaphragm. For this reason the photocell data can be relied on to give only an approximate value for the incident energy on the embryo.

Since the photocell and quartz microscope transmission measurements give a better measure of the incident energy on the embryo, the efficiency curves obtained by correction with the photocell data will be considered in the comparison with absorption spectra. Although the curves drawn between the points determined by interpolation are based upon the assumption that the morphological effect is proportional to dose, this does not influence the magnitude of the absorption maxima and minima since the two experimental points are close together at most wave lengths.

As was pointed out in the first part of the discussion, the significance of the relative photochemical efficiency curves lies in the fact that they can be used to determine the chemical nature of the irradiated material. This is possible since photochemical efficiency curves are an indirect measure of the amount of energy absorbed when certain fundamental assumptions are fulfilled. These assumptions are: (1) radiation must be transmitted by the absorbing system; (2) the quantum yield for the substance absorbing the energy must be the same for all effective wave lengths; and (3) only the radiation which is absorbed and involved in producing the photochemical effect is measured. The validity of this technique was proved both mathematically and experimentally by Warburg (1927) and (1930). Since then, several investigators, namely, Gates (1930), Oster (1935), Giese (1938), and Landen and Uber (1939), have used this method.

An examination of the validity of the data in the present investigation with reference to the three assumptions shows that the first assumption is supported by two types of evidence. From cross-section measurements of the notochord, it appears that radiation affects its shape. Since the notochord lies directly beneath the neural plate, it is suggested that radiation is transmitted by the neural plate. This type of reasoning, however, is subject to the criticism that the notochord might be affected indirectly through the action of radiation on the neural plate or other structures. More conclusive evidence is presented by the dark and disorganized appearance of the mesenchyme cells beneath the neural plate.

From the experiments in this study data concerning the constancy of quantum yield with wave length are not available. In order to ascertain the quantum yield of the material engaged in folding it would be necessary to know the particular compound involved. In this investigation no attempt is made to determine the exact chemical compound but only to ascertain to what general group of compounds the material belongs. It is interesting to note that Harris, Bunker, and Mosher (1938) found the quantum yield for ergosterol to be constant for wave lengths 2537, 2652, 2894, 2967, and 3025 Å. Bunker, Harris, and Mosher (1940) repeated this experiment for 7-dehydrocholesterol and found a uniform quantum efficiency for all wave lengths except 2967 Å which was slightly more efficient. In a study of proteins, Landen (1940) and Hollaender and Duggar (1936) found the quantum yield to be constant for wave lengths between 2400 and 3130 Å.

Evidence for fulfillment of the third assumption will now be considered. As shown in unpublished work by the author, inhibition of the folding process is almost exclusively the result of an effect upon the neural plate cells. Furthermore, a very small amount of neural plate material seems to be affected by the energy involved in inhibiting folding since effects on mitosis and volume of the neural plate do not appear to influence the folding process. Consequently, it can be concluded that if other molecules are absorbing some of the incident energy required to prevent closure of the neural tube, the amount absorbed must be very small. The incident dose at wave length 2804 Å required to produce the morphological effect on the neural tube is approximately 71 ergs/mm.^{2}. When the incident energy is expressed in quanta, a value of 1.1×10^{13} quanta/mm.^{2} is obtained. If this figure is divided by the number of cells in a square millimeter of the neural plate which is of the order of 10^3 to 10^4, a value of 1.1×10^9 to 10^{10} quanta is obtained for the incident energy on one cell. This is a smaller dose than that required to produce just a perceptible retardation of cleavage in one sea urchin egg. Giese (1938) found that an incident energy of 623.36 ergs/mm.^{2} or 3.74×10^{11} quanta per egg at wave length 2537 Å was the smallest dose which would retard cleavage and that wave length 2804 Å was only twice as effective as wave length 2537 Å. In view of this, it can be safely concluded that no more than a very small amount of energy is absorbed by molecules "screening" the inactivated material.

Since this technique is valid within the limitations of the data, it is possible to compare the relative photochemical efficiency curves with the absorption spectra of chemical compounds. A very extensive series of absorption spectra of the most important biological substances was compiled from the data of several hundred investigators and published by Ellinger (1937; 1938). The major groups of compounds included were fats, carbohydrates, proteins, sterols, phosphatides, carbonic acid and its derivatives, alkaloids, and glycosides. The photochemical efficiency curves obtained in this study possess absorption maxima at wave lengths 2576 and 2804 Å and a minimum at wave lengths 2650–2700 Å. Comparison of these curves with the absorption spectra of biological compounds shows that the efficiency curves resemble very closely the absorption spectra of certain sterols. The sterol curves which are very similar to the efficiency curves are for three vitamin D precursors, namely, 7-dehydrocholesterol, ergosterol, and lumisterol. In Figure 6, the photochemical efficiency curve for inhibition of closure of the neural tube for a distance of at least one-third its length when correction is made for absorption by the vitel-

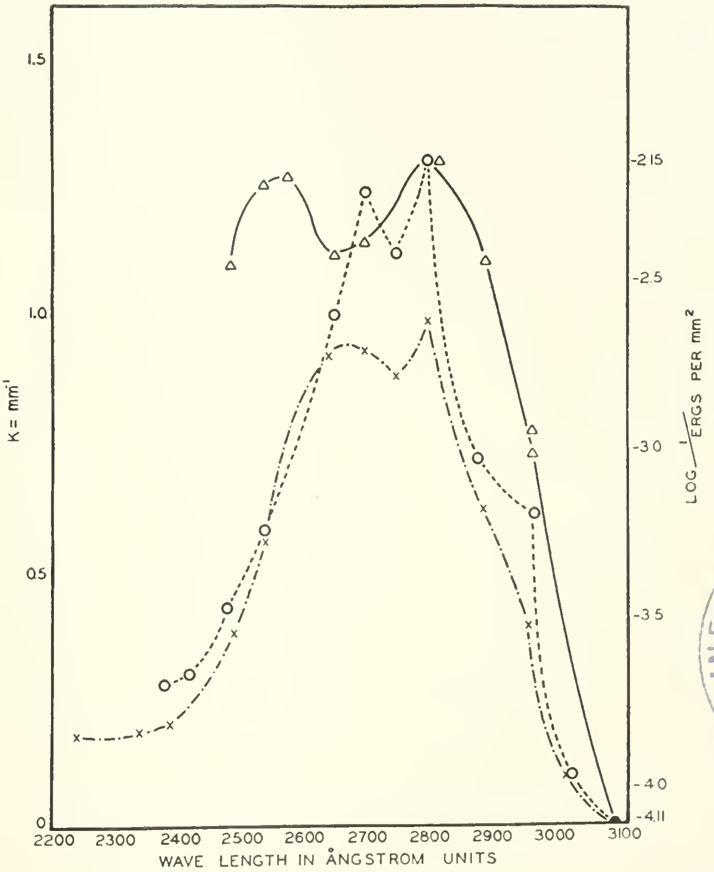

FIGURE 6. The photochemical efficiency curve for inhibition of the folding process in neural tube formation (—△—) compared with the absorption spectra of lumisterol (—·—×—·—) and 7-dehydrocholesterol (---○---).

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line membrane based upon the quartz microscope and photocell data is compared with the absorption spectra of two of these vitamin D precursors.

No attempt is made to compare the efficiency curve with a particular sterol curve since absorption curves of very closely related compounds are known to vary, particularly in the position of the maxima. Von Dimroth (1939) found that the absorption spectra of sterols are dependent upon the number and location of double bonds. The position of the absorption maxima is dependent also upon whether the double bonds are located in one or in two rings. Furthermore, the solvent and pH of the mixture play a role in the position of the absorption bands. It is also possible that the chemical material which has been irradiated is an unidentified sterol or group of sterols. Consequently, an exact match of the curves cannot be expected.

Attention is called to the fact that the photochemical efficiency curves do not resemble absorption spectra of single proteins. Most protein curves have only one maximum which is at 2800 Å and a minimum around 2500 Å which is the position of the secondary maximum in the efficiency curve presented in Figure 6. It should be mentioned, however, that a mixture of nuclear and cytoplasmic proteins might give an absorption curve similar to the photochemical efficiency curves. This is unlikely because it would involve the photochemical inactivation of two different compounds simultaneously. Furthermore, nucleoproteins which are involved in the mitotic mechanism do not seem to be involved in folding because the wave lengths which are most effective in inhibiting mitosis are least effective in inhibiting folding.

The part that sterols play in early development is discussed by Needham (1942). He calls attention to their possible role in development as "neurogens," i.e. substances which stimulate gastrula ectoderm to neural differentiation. From the present investigation it is evident that sterols are very probably involved at a slightly later stage in development, namely, in neural tube formation. Although the technique used in this study does not allow a final or conclusive identification of a specific compound to be made, it does give (1) evidence that a special compound is involved in folding that does not seem to be engaged in other morphogenetic processes at this time in development and (2) a very strong indication as to the chemical nature of this material.

SUMMARY

1. Chick embryos ranging in age from the primitive streak to the 8-somite stage were irradiated with monochromatic ultraviolet radiation of wave lengths 2483 to 3130 Å and subsequently incubated for 30 hours.

2. Histological studies show that radiation inhibits the folding process in neural tube formation, while cell division and volume changes continue. This effect on the neural plate is uniform for all wave lengths except 2967 Å; nevertheless, wave length 2967 Å inhibits folding.

3. After correction for absorption by the vitelline membrane, the incident energy on the embryos required to inhibit the folding process was calculated. Folding is affected by all wave lengths except 3130 Å and photochemical efficiency curves for the folding process are presented.

4. In order to obtain information concerning the chemical nature of the mate-

rial involved in folding, the photochemical efficiency curves which are an indirect measure of absorption were compared to the absorption spectra of biological compounds. The validity of this technique is based upon three fundamental assumptions which are satisfied within the limitations of the available data.

5. The photochemical efficiency curves are very similar to the absorption spectra of sterols, particularly vitamin D precursors. The small doses used in the inhibition of folding and the high sensitivity of sterols to ultraviolet light add support to the finding made with absorption measurements that sterols are involved in the folding process.

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SEROLOGICAL RELATIONSHIPS BETWEEN THE MOLLUSCA AND OTHER INVERTEBRATES[1]

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INTRODUCTION

Until recently, opinions regarding classification, interrelationships and phylogeny of organisms have depended primarily on interpretations derived from morphological and developmental data. As a result of the subjective nature of such interpretations, concepts have differed and considerable controversy has arisen concerning the phylogenetic status of certain forms and even of entire groups of organisms. In addition to their use in diagnosis and control of many diseases, serological methods have, since the turn of the century, served as an additional source of information concerning the phylogenetic relationships of plants and of animals.

Of the several types of serological reactions available the precipitin test is most suitable for the determination of interrelationships of organisms. Discovered by Kraus (1897), the precipitin reaction was thought to be specific, since the blood sera of goats which had been inoculated with sterile cholera, typhoid or plague culture filtrates caused precipitates only when mixed with the particular bacterial filtrate used for immunization.

That foreign proteins other than bacterial ones were antigenic and could cause the the appearance of precipitins in the blood of an injected animal became evident from the research of Bordet (1899) and of Tchistovitch (1899), working independently. Tchistovitch observed that the serum of rabbits which had been injected with horse or eel serum caused the precipitation of the antigen identical with that used in its production, and, therefore, he claimed that the reaction was specific. Of greater biological significance were the results and conclusions of Bordet, who discovered that antichicken serum, produced in the rabbit, reacted not only with chicken serum but also with pigeon serum, although much less strongly in the latter case; he thus concluded that the precipitin reaction is not strictly specific. These observations have been confirmed and extended by other, more recent researches.

The independent discovery by Uhlenhuth (1901) that the precipitin reaction is not strictly specific, since an antiserum against one protein may react with other closely related proteins as well as with the protein used in its production, is the basis of the application of serological reactions to biological problems involving interrelationships of organisms. He concluded that precipitin reactions are quantitatively, as well as qualitatively, specific, i.e., any antiserum will react more strongly with the antigenic substance used in its formation (homologous reaction) than with other antigenically different, though closely related, substances (heterologous reac-

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[1] This research was conducted with the aid of funds supplied by the University of Missouri Research Council.

tions), the degree of relationship being indicated by the strength of the heterologous reactions as compared to the homologous one.

Ascoli (1902), Graham-Smith and Sanger (1903), and Fornet and Müller (1908, 1910) improved upon the flocculation type of precipitin technique by carefully introducing the antiserum into the precipitin tube below the antigen so that a definite interfacial boundary was maintained; at the interphase between the two reagents a layer or "ring" of precipitate formed and the end-point or titer could be determined with greater precision.

To Nuttall (1904) belongs the credit for the first extensive application of the precipitin reaction to the problem of animal relationships or phylogeny. Several thousand qualitative and quantitative reactions were performed with sera of approximately six hundred species of animals, and the degree of reaction between any particular antiserum and several different antigens was found to be in proportion to the degree of relationship between the animals concerned, i.e., the results of the precipitin tests confirmed, in general, the classification and relationships to the animals based on morphology and development.

During the past two decades, the researches of Professor Alan Boyden and his students have yielded outstanding contributions not only to our knowledge of animal relationships, particularly among the vertebrates, but also, and more significantly, to refinements in techniques and methods. Boyden (1926), employing the "ring" technique, obtained confirmation of his quantitative reactions and titers by reciprocal tests, the importance of which he summarized by stating that "it would seem then that this principle of reciprocal relationships could be used as a test of the truth of the values obtained in the precipitin reaction. . . . Only those values which check within the limits of error of the reaction may then be taken." More recently, by employing a highly sensitive instrument, the photronreflectometer (cf. Libby, 1938), for measuring turbidities developed by the precipitin reaction (flocculation technique), Boyden and his students (Chestnut, DeFalco, Gemeroy, Leone) have approached with even greater objectivity the solution of relationship problems within restricted groups of animals.

Serological relationships indicate relative physiological and chemical affinities, and presumably, therefore, genetic and phylogenetic relationships, since the protein constitution is the basis for morphological as well as physiological characteristics. With the advent of and improvement upon the precipitin reaction an *objective* method was provided for solving problems of phylogenetic relationships. However, until recently, the variations in results of precipitin reactions in experiments designed to aid in phylogenetic studies were so great that one could not be sure of interpretations which the results may have suggested.

Mez and his students have based a phylogenetic tree of plants upon the results of precipitin reactions and reported agreement between such relationships and those derived from morphological considerations. Zoologists have not yet constructed phylogenetic trees based solely upon results of serological tests. At the present state of our serological information, construction of such trees would be impossible, but, with the improvements in and greater uniformity of techniques and with the accumulation and interpretation of data, such attempts may be made.

The relationship of the Mollusca to other invertebrates has long been the source for considerable speculation. Embryological evidence would indicate that annelids, arthropods and mollusks are related by reason of (1) the fate of the blastopore which becomes the mouth, placing these animals in a group known as Proterostomia, (2) the formation of the mesoderm which is by proliferation from two mesoblastic cells, the mesodermal bands later splitting to form schizocoelous coelomic spaces, and (3) the type of cleavage, which is, generally speaking, determinant.

The present paper will report some experiments involving precipitin reactions designed to determine the serological relationship of the Mollusca to other invertebrates.

Material and Methods

Antigens were prepared from representatives of four invertebrate phyla: Annelida, *Nereis virens;* Arthropoda, *Limulus polyphemus;* Mollusca, *Busycon carica, Busycon canaliculatum, Pecten irradians;* Echinodermata, *Asterias forbesi.* It will be noted that the habitat of all these animals is marine.

Preparation of antigen

Preparation of antigens was essentially similar to that reported in earlier publications (Wilhelmi, 1940, 1942). Although numerous difficulties were encountered, particularly in the extraction of annelid and molluscan materials, all antigens are extracts of tissues.

Following a rather long preliminary period of exposure to filtered sea water to remove certain contaminating organisms and debris, the annelids were washed through several changes of sterile sea water contained in large, covered crystallizing dishes and finally rapidly through sterile distilled water to remove excess salts. In the case of Limulus and the mollusks, following the preliminary treatment of the specimens with filtered sea water and washing with 70 per cent alcohol, exoskeletal structures were removed. Asterias specimens were treated in a similar manner, but before opening specimens the tips of the arms were cut and the coelomic fluid drained into a sterile receptacle to be frozen in dry ice; then the aboral surfaces of the arms were removed exposing the inner soft tissues. Antigens of the respective animals were prepared by extracting the soft tissues, exclusive of the digestive tract. Materials were handled independently and manipulated carefully to avoid contamination.

Tissues of the living animals were minced on a glass plate or in a shallow (Petri) dish and, after placing in a sterile container, frozen rapidly by means of dry ice. When frozen, the material was placed in a vacuum desiccator, evacuated by a Cenco-Hyvac pump, over drierite (anhydrous calcium sulfate) until thoroughly dried. By this method the native chemical structure of antigenic substance is preserved, since the rapid freezing does not permit deterioration or denaturation of constituent chemicals. The high vacuum causes such rapid evaporation of water that substances remain frozen, the dehydration is more rapid, and oxygen, which might alter the antigens, is removed. Desiccation in the frozen state usually results in dried material with a spongy texture which renders it easy to pulverize. However, in the present experiments considerable difficulty was experienced in effecting complete desiccation of the annelid and molluscan materials in the 24-hour period usually required by the process.

When the material appeared to be completely desiccated, it was triturated, using a mortar and pestle, and then returned to the desiccator under vacuum for an additional 48 hours or until extractions were to be made.

Two types of antigens were prepared from the desiccated tissues. One type was a saline extract of the entire animal, and the other, a saline extract of the lipid-free materials of the animal. To prepare the first, desiccated material was weighed and then extracted for 12 to 24 hours with sterile, buffered saline solution maintaining a pH of 7.3, one part by weight of dried substance being mixed with ten parts by volume of extractant. Extraction was done at room temperatures in sterile Erlenmeyer flasks under constant agitation provided by a mechanical shaker. To prepare the second type of antigen, the lipids were removed from the material by repeated extractions with Bloor's mixture, consisting of three parts absolute ether to one part absolute alcohol, one part by weight of powdered material being mixed with one hundred parts by volume of Bloor's mixture. After weighing the lipid-free residue, it was extracted with sterile buffered saline solution using the same methods as employed to obtain saline extracts of the entire animal.

After aqueous extraction the material was filtered through sterilized Seitz filters, and the clear, sterile filtrate was transferred to sterile, rubber-stoppered vaccine bottles and kept in the refrigerator until needed for injections. The residue from the aqueous extraction was desiccated and weighed, and the exact amount of material in solution was determined by calculating the difference in weight of residues before and after the aqueous extraction.

Antibody production

For injection purposes, healthy rabbits of equivalent weights were used following a preliminary period of observation. For the experiments reported herein, a longer method of antibody production was employed than that reported in earlier papers. Each rabbit received eight intravenous injections, one every other day.

In the series of eight injections, a total of 500 mg. of dry-weight antigen was injected. The 500 mg. was divided into doses of increasing amounts, i.e., approximately 25, 50, 50, 50, 75, 75, 75 and 100 mg., respectively.

Ten to 14 days after the last injection, exsanguination of the rabbit was effected by aseptic, intracardial puncture, using a large, sterile hypodermic syringe fitted with a 19-gauge needle, and the blood was placed in large (250 cc.), sterile, cotton-plugged centrifuge tubes and allowed to clot at room temperatures. After standing in the refrigerator for several hours, the clotted blood was centrifuged; the serum was drawn off and used immediately for tests or filtered through a sterile Seitz filter and placed in sterile vaccine bottles for storage in the refrigerator until needed for the precipitin reaction.

Precipitin test

In a precipitin tube (8.0 mm., inside diameter), 0.5 cc. of undiluted antiserum was carefully overlaid by 0.5 cc. of appropriately diluted test antigen so that a definite interfacial boundary between the two reagents was maintained. The antigen was brought to the proper concentration by adding sterile saline solution buffered to a pH of 7.3. For most tests a standard dilution of each test antigen was prepared so that 1.0 gram of dry-weight material, either whole-animal or lipid-free (depending upon which was needed), would be contained in 100 cc. of saline solution, i.e., 0.01 g./cc. or a dilution of 1 : 100. In homologous tests this standard was diluted to 1 : 1000 for the initial tube, and the dilution in each succeeding tube of a series was doubled, so that a series of antigen dilutions from 1 : 1000, through 1 : 2000, 1 : 4000, etc. up to 1 : 2,048,000 was employed. To make some of the heterologous precipitin tests it was necessary to evaporate the test solutions to the desired antigenic concentrations, evaporation being effected by placing the extract in sterile Petri dishes in a partial vacuum over drierite. A dilution of 1 ; 10 was employed in the initial tube, and the concentration of antigen was halved in each successive tube. In a positive reaction, a "ring" or layer of white precipitate occurred at the interphase of the antiserum and antigen.

The titer of a reaction is the highest antigen dilution which yields a visible precipitate within one hour at 37° C. when tested with an antiserum, either homologous or heterologous, the amount of antigen being determined on the basis of dry weight of antigen material actually in solution. In all cases the readings and titers were taken at the end of one hour at 37° C.

Control tests were always conducted and involved the use of (1) normal sera and antigen solutions, (2) immune sera and the extracting salt solution, and (3) normal sera and the extractant.

OBSERVATIONS AND RESULTS

Table I presents the titers of both homologous and heterologous reactions between the several species of invertebrates employed in these experiments. Each titer represents the average of at least four, and in many cases six or eight, determinations at 37° C. Negative reactions are indicated by minus signs (—), and tests which were not performed, by blank spaces. The letters *w* and *e* refer to whole-animal and lipid-free materials, respectively.

The titers of homologous tests cover a wide range, from 1 : 49,000 to 1 : 512,000, results from the use of lipid-free antigens being the only ones considered reliable. Under the conditions of these experiments, the species-specific titer may be designated as 1 : 128,000. However, in two species (*Busycon carica* and *Asterias forbesi*) the titers did not fall within the limit of experimental error (plus or minus one dilution tube) allowed in the precipitin reaction, although in each case the titer was only one dilution tube removed from the accepted limit of error. The titers of heterologous reactions never exceeded those of homologous ones.

In general, reciprocal tests confirmed the original ones, i.e., were of the same order of magnitude. Such confirmation is imperative in experiments designed to determine phylogenetic relationships by serological methods.

Particular attention is directed to the titers of homologous and heterologous reactions when whole-animal extracts were used as antigens. It will be noted that the variability is exceedingly great and that several inconsistencies appear, particularly in the heterologous tests; for example, the titer (1 : 4000) of the reaction involving antiserum against *Limulus polyphemus* whole-animal materials and antigen prepared by extracting whole *Busycon carica* is higher than that (1 : 1000 or 1 : 2000) between *Pecten irradians* and *Busycon carica* materials, although Pecten is undoubtedly more closely related to Busycon than is Limulus. As a matter of fact, the only cross-reactions between arthropod and molluscan materials occurred

Table I

Homologous and heterologous precipitin reactions

(Number in parenthesis below certain titers indicates relationship expressed as per cent value of homologous titer)

Antiserum / Antigen		*Nereis virens*	*Limulus polyphemus*		*Busycon carica*		*Busycon canaliculatum*		*Pecten irradians*	*Asterias forbesi*
		e	w	e	w	e	w	e	e	e
Nereis virens	e	*106,000* (*100*)	—	1,000 (0.78)		— (0.000)	—	25 (0.0195)		100 (0.0195)
Limulus polyphemus	w		64,000		400					
	e	500 (0.47)	16,000	*128,000* (*100*)	—	— (0.000)		— (0.000)	— (0.000)	— (0.000)
Busycon carica	w		4,000		16,000					
	e	10 (0.0094)	—		2,000	*49,000* (*100*)		49,000 (36)	2,000 (1.887)	— (0.000)
Busycon canaliculatum	w		1,000		8,000		24,000			
	e	40 (0.038)	—	— (0.000)	2,000	13,333 (27.2)	6,000	*128,000* (*100*)		
Pecten irradians	e	— (0.000)	—	— (0.000)		1,000 (2.04)		1,000 (0.78)	*106,000* (*100*)	— (0.000)
Asterias forbesi	e	40 (0.038)	—	— (0.000)		— (0.000)	—	— (0.000)	— (0.000)	*512,000* (*100*)

when whole-animal materials had been utilized as antigens in preparation of the antisera. Other interesting comparisons can be made by reference to the Table.

The figures in parenthesis represent the titration data converted to percentages of the homologous reaction titers. It will be noted that percentages were calculated only for those reactions involving lipid-free materials. The homologous titer of a particular antiserum is taken as 100 per cent and the titers of all heterologous tests against that antiserum are expressed as percentages of the homologous titers. For example, *Asterias forbesi* antigen when tested against *Nereis virens* antiserum yields a titer of 1:40; the homologous titer of *Nereis virens* antiserum being 1:106,000, the percentage value becomes $\frac{40}{106{,}000}$ or 0.038 per cent.

Discussion

Homologous reactions of high titer were thought necessary if heterologous reactions between such distantly related groups as the Mollusca and other invertebrate phyla were to be obtained. In a series of preliminary experiments in which the same method of producing antibodies was used as was reported previously (Wilhelmi, 1942), i.e., the short method of antibody production, all precipitin tests between materials of Mollusca and other invertebrate phyla were negative, although the homologous reactions yielded titers which were higher and more consistent than those reported herein. Since Wolfe (1935) had reported that the titers of heterologous reactions could be increased by extending the series of injections of antigen over a longer period of time, a longer method of antibody production was attempted. The fact that the homologous titer is lower than in previous experiments is a confirmation of Wolfe's results.

Since positive reactions were not obtained in the preliminary experiments mentioned above, in addition to extending the period of injections, whole-animal extracts, as well as lipid-free materials, were employed as antigens, since in the preliminary tests only lipid-free antigenic materials had been used. The purpose of the experiments with lipid-containing antigens was to ascertain whether, in case of failure to produce cross-reactions with lipid-free antigens, positive interphylar tests between Mollusca and materials of other invertebrate phyla could be obtained under *any* circumstances or conditions. It will be recalled that the only cross-reactions between arthropod and molluscan materials occurred when whole-animal materials had been utilized as antigens in the preparation of antisera. From earlier research and from reports by other authors, Wilhelmi (1942) had concluded that, although the zoological relationships of organisms are closely paralleled by the serological and chemical properties of their respective proteins, i.e., the more distant the phylogenetic relationships of organisms, the greater the difference between their constituent proteins, the lipids of distantly related species may be very similar, if not identical, immunochemically. In the experiments reported in that paper, lipids had not been employed as antigens in direct tests, but the present experiments lend ample support to the viewpoint that lipids of distantly related species may be very similar immunochemically. It is very apparent that the specificity of antisera produced by injection of lipid-containing antigens is dependent not upon the proteins contained therein but is controlled by the lipids present. That is to say, although the arthropod and molluscan proteins are not closely enough related chemically to produce cross-reactions, their haptenic lipids, rendered antigenic when in combination with proteins, are immunochemically so similar that they produce antisera which will yield positive precipitin reactions even at relatively high dilutions. In view of the results of these tests, of the observation of Boyden (1936) in research upon Crustacea that "*the convergent reactions obtained with antisera to native serum . . . disappeared* following ether extraction," and of the conclusion by Cumley (1939) from experiments with *Drosophila* spp. that the presence of ether-soluble material in antigens interferes with the reliability of the reactions, it may now be stated that, in all experiments designed to determine the phylogenetic relationships of organisms by means of serological methods, antigens must be prepared from lipid-free materials. Since antisera prepared by injection of lipid-containing antigens yield unpredictable, inconsistent and divergent results, the inescapable conclusion is that, in all experiments dealing with serological relationships of organisms, demonstrable relationships are of doubtful value and resulting interpretations questionable unless experiments are performed with lipid-free materials. Lipid-free antigens, whether the source is blood sera or the various body tissues and organs of organisms, must be prepared and used in all relationship studies employing the precipitin "ring" test.

The adult morphology of the Mollusca yields very little in the way of satisfactory clues to the phylogeny of the group, although the arrangement of the ganglia of the central nervous system may represent a palingenetic structural organization on a segmental plan. In Amphineura and some of the lower Gastropoda there is a ladder-like nervous system, resembling that of some Turbellaria and of the most primitive worm-like arthropods (Peripatus), and a condition considered by some to be a pseudometameric arrangement of organs. When one studies the embryological development in the group, the affinities of the mollusks to other proterostomian invertebrates become apparent because of the facts that (1) cleavage is of the determinant type, (2) the blastopore becomes the mouth, (3) the mesoderm is formed by proliferation from two mesoblastic cells, (4) the coelomic spaces arise by schizocoelous methods, and (5) the trochophore, or a modification, the veliger, larva occurs in the life cycle. Lameere (1932) regarded the Mollusca as derived from the nereidiform annelids near the Amphinoinidae. However, Nierstrasz and Stork (1940) contend that they are not phylogenetically closely related to the annelids, but that their organization is more readily comparable with that of the Turbellaria, especially with respect to the tendency towards pseudometamerism and the structure of body wall and nervous system. Parker and Haswell (1930) argue that the occurrence of the trochophore larva in the life cycle of the Mollusca need not necessarily be regarded as evidence of their derivation from the Annelida. "In fact the absence of segmental repetition of parts in all, with the exception of Nautilus, would seem to indicate the derivation of the phylum from a group in which metamerism had not arisen. It will be readily recognized that the gap between the typical trochophore and certain forms of Turbellarian larvae (Müller's larva) is not a very wide one, and might be covered by adaptation of the larval Flat-worm to a freer pelagic life. If we were to suppose that the most primitive Mollusca were derived from Turbellarian-like ancestors, the conversion of a larva of the type of Müller's larva into a larval form like the molluscan trochophore would also have to be postulated. This might involve a common platyhelminth origin for Annulata and Mollusca with subsequent extreme divergence—a divergence in which the respective trochophores would take part, though in a limited degree."

The results of the present experiments support the conclusion that the Mollusca are serologically more closely related to the Annelida than to any other invertebrate phylum tested, since interphylar reactions did not occur between the representative Mollusca and those of any other phylum except the Annelida when lipid-free antigenic materials were employed to produce antisera and to make the precipitin test. Under the conditions of the experiments annelid-molluscan titers were of the same order of magnitude as annelid-echinoderm titers. However, it must be remembered that, in order to produce reactions between molluscan materials and those of other proterostomian phyla, the longer method of antiserum production had to be used and, as pointed out by Wolfe, such antisera are less specific and give greater group reactions. The fact that no positive reactions were noted in molluscan-arthropod tests is in agreement with results of intradermal tests performed by Tuft and Blumstein (1940) in which it was found that neutralization with one of the members of the Mollusca obliterated reactions to all other members of that group but not to those of the Crustacea. Thus, animals of the Mollusca group and those of the Crustacea group bear no antigenic relationship to each other; they concluded that there is a common antigen among Mollusca and that a complex molecule with several antigenic fractions is present among the Crustacea. In the present experiments intraphylar tests between the molluscan species were done in order that a reference point would be available for interpretation of the interphylar reactions. The values 36 per cent and 27.2 per cent, representing reciprocal values of tests between materials involving the two species of Busycon (*B. carica* and *B. canaliculatum*), are not of the same order of magnitude as the percentage value (85 per cent) reported by Boyden (1943) for members of the same genus, but more nearly approach his values for members of closely related families; however, his data were obtained by the use of the photronreflectometer upon vertebrate materials and perhaps, therefore, are not strictly comparable. In the same paper, Boyden, in reference to unpublished research by Chestnut, reports that the photron'er has been, or is being applied, to the study of Mollusca. Makino (1934), by means of precipitin (flocculation), complement fixation and anaphylactic reactions, was able to differentiate the various genera of Mollusca which he employed and to group them according to their relationships. In the present experiments, the tests between *Busycon carica* antigen and *Pecten irradians* antiserum yielded a relationship value of 1.887 per cent, and the reciprocal tests, 2.04 per cent, so that interclass tests among Mollusca presumably yield percentages in the vicinity of 1 per cent to 3 per cent. Interphylar values are all less than 0.78 per cent, all between Mollusca and other invertebrate phyla being less than 0.04 per cent.

It is apparent that serologically, as well as developmentally, the Mollusca are more closely related to the Annelida than to any other group of invertebrates tested. It should be emphasized, however, that the results of these precipitin tests need not be interpreted to mean that the Mollusca arose from Annelida, since the precipitin reaction is a measure of *present* chemical relationship of the antigenic constituents of the protoplasm of existing species. These reactions may be interpreted to indicate that the Mollusca evolved from animals which also gave rise to present-day Annelida, and perhaps a Turbellarian ancestry such as proposed by Parker and Haswell (1930) will be substantiated by future research and investigation.

SUMMARY AND CONCLUSIONS

The precipitin reaction was applied to the problem of the relationship of the Mollusca to other invertebrate phyla. From the results, it is apparent that serologically, as well as developmentally, the Mollusca are more closely related to the Annelida than to any other group of invertebrates when lipid-free materials are employed as antigens. The results indicate that Mollusca evolved from animals which also gave rise to present-day Annelida.

Consistent results, confirmed by reciprocal tests, were obtained by injection of aqueous solutions containing 500 mg. of dried antigenic material from which the lipids had been removed. Injection procedure involved eight intravenous injections of antigen on alternate days and with gradually increasing dosage. To test the influence of the lipids, one group of experiments involved the use of whole-animal, i.e., lipid-containing antigens. The inconsistent and divergent results of the use of antisera resulting from these antigens dictate that, in relationship studies, whether performed with tissue extracts or blood sera, it is essential that lipid-free materials be used in order to obtain accurate and reliable results and to preclude the possibility of group reactions.

With two exceptions, the titer of homologous reactions is 1:128,000 plus or minus one dilution tube, and heterologous titers never exceeded homologous ones.

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BIOCHEMICAL FACTORS IN THE MAXIMAL GROWTH OF TETRAHYMENA[1]

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INTRODUCTION

The earliest attempt to elucidate the nutritional requirements of the ciliate *Tetrahymena geleii* (*Glaucoma piriformis*) by the pure culture technique was made by Lwoff (1924). At that time the failure of the ciliate to grow in solutions of pure amino acids was attributed to a lack of specific chemical supplements. Later Lwoff (1932) suggested that a requirement for polypeptides was responsible for the lack of growth in such media.

This early work indicates immediately that the problem of the nutrition of *T. geleii* is a dual one. No investigation of the nitrogen requirements may be made without some knowledge of the supplementary factors needed. So far it has been shown that thiamine is important if not absolutely necessary to the nutrition of *T. geleii,* while riboflavin, pantothenic acid, nicotinic acid and pyridoxine probably play a part (Elliott, 1935b, 1939; Lwoff and Lwoff, 1937, 1938; Hall, 1940a, b, 1942; Baker and Johnson, 1941; Kidder and Dewey, 1942). In a preliminary report Dewey (1941) indicated that other factors of unknown nature are required for maximal growth. A great deal less work has been done on the nitrogen nutrition (Elliott, 1935a; Hall and Elliott, 1935; Dewey, 1941; Hall, 1942). None of the results of these investigations is conclusive. The report of Kline (1943) that *T. geleii* (*Colpidium striatum*) will grow in an amino acid solu-requires confirmation.

An attempt to obtain knowledge of the supplementary requirements necessitates the use of a basic medium capable of supplying the nitrogen and carbon needs of the organism and ideally completely lacking in supplementary factors; the testing of all known growth promoting substances, and the search for and purification of possible unknown growth factors.

I take pleasure in acknowledging my indebtedness to Professor G. W. Kidder of Brown University for his invaluable advice and encouragement. Thanks are due to Dr. R. J. Williams for a gift of pure calcium pantothenate and to Dr. Samuel Lepkovsky for the gift of a concentrate of his factor I.

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MATERIAL AND METHODS

Preliminary experiments were carried out using four strains of *Tetrahymena geleii* and one strain of *T. vorax*. Since only quantitative differences were found between these strains, later work was confined to strain W (Claff, 1940; Kidder, 1941a). This strain was selected because it showed the most rapid growth rate and the greatest resistance to increased salt concentration of the medium. Certain experiments were also carried out using strain H, obtained from Dr. R. H. Hall.

All experimental media were prepared with water distilled twice in an all-Pyrex still over potassium permanganate and all the glassware used was Pyrex. Glassware was cleaned by soaking for at least one hour in a hot saturated solution of trisodium phosphate or a preparation sold commercially as "Keego," followed by careful rinsing to remove all traces of the cleaning agent.

Stock cultures were maintained in a 2 per cent solution of Difco proteose-peptone and also for a time in a one per cent solution of crude casein (Eimer and Amend). The fact that these ciliates could be maintained in solutions of crude casein suggested the use of a highly purified casein as a basic medium. Casein Harris (highest chemical purity) was used as a one per cent solution. Although this preparation has been called "vitamin-free" casein, this term is somewhat misleading in that traces of certain growth substances appear to be present in amounts sufficient to affect the growth of protozoa. It is, however, still adequate as a basic medium for testing responses to growth stimulants because the growth in it alone is still far from optimal with regard either to the rate of growth or the size of the population supported. For the purposes of this paper the growth in crude casein is regarded as being optimal.

Since casein, in order to be available to the ciliates, must be in solution or at least colloidal suspension, the following method was used to disperse it. To the casein was added sufficient alcohol to completely wet it, then water containing 1.0 to 1.5 ml. of normal NaOH per gram of casein was poured in slowly with stirring. With vitamin-free casein it was found necessary to add a balanced salt solution in order to obtain growth. For this purpose the modified Osterhout solution employed by Barker and Taylor (1931) was used in all but the first series of experiments. The concentrated stock solutions were added to the water used to make up the medium. The suspension of casein was allowed to stand with occasional stirring until solution was complete. Then normal HCl was added drop by drop with stirring between drops until the alkali had been neutralized. Care must be taken to add the acid slowly in order to prevent precipitation of the casein. The reaction of the medium was adjusted to pH 6.8–pH 7.0. The medium was then dispensed into tubes in amounts of 5 ml., plugged with cotton and autoclaved for 15 min. at 15 lbs. pressure. Elliott's (1939) report that vitamin-free casein required digestion with pepsin before it could be utilized by the ciliates is possibly due to a failure to put the casein into solution.

Experimental cultures were examined for growth, the results recorded and transplants made at intervals of 48 hours in casein media. The interval was slightly longer in gelatin media. The tubes were kept, however, for from one to two weeks and re-examined at intervals. Such a procedure gives results which indicate the presence or absence of factors necessary to maintain growth at or near a maximum rate. These results cannot, of course, be compared with those obtained by incubating cultures for a week or more before examination, since by that time a slowly growing culture may have reached a concentration equal to that of a rapidly growing culture. In order to eliminate the effects of carry-over only the results of the third transplant in a given medium are considered. Growth is recorded as zero to four plus by comparison with growth in a control medium. Growth recorded as zero may indicate survival of the inoculum or an increase of one or two divisions, while four plus growth represents a population of from 75,000 to 100,000 organisms per ml. Two or three plus growth is intermediate. The cultures were kept at room temperature (20°–22° C.). Transplants were made using an open bacteriological loop and ordinary bacteriological technique.

Cultures were also incubated in Kidder culture flasks. The third transplant in a tube was inoculated into the flask and the growth followed by making counts at intervals of 12 or 24 hours (Kidder, 1941a).

Gelatin in concentrations of 1 per cent, 1.5 per cent and 2 per cent was also used in certain experiments. Both Harris gelatin (vitamin-free) and Eastman de-ashed gelatin were used. Another medium consisted of 1 per cent silk peptone (Seidenpepton, Hoffman-LeRoche). These media were in some cases supplemented with amino acids in various concentrations as well as with various growth promoting substances. In other cases solutions of the pure amino acids alone were used. The following amino acids were obtained from the Eastman Kodak Co.: l-histidine, l-leucine, dl-threonine, dl-β-phenylalanine, dl-methionine, d-arginine carbonate and d-lysine hydrochloride. From the Hoffman-LaRoche Co. l-tryptophane, d-isoleucine, dl-valine and glycine were obtained and from Eimer and Amend, tyrosine.

The basic medium was supplemented with vitamins and growth factors of known chemical composition as well as with crude extracts of animal and plant material. The known compounds were supplied (with one exception to be noted later) at a level of 0.001 mg. per ml. except in the case of i-inositol, which was used in a concentration of 0.004 mg. per ml., and biotin, which was used in a concentration of 0.00008 mg. per ml. Thiamine hydrochloride and riboflavin were obtained from the Hoffman-LaRoche Co. A sample of calcium pantothenate was obtained from Dr. R. J. Williams and subsequent calcium pantothenate as well as biotin methyl ester from the S. M. A. Corp. Pyridoxin hydrochloride (first used as factor I concentrate of Lepkovsky) was obtained from Merck and Co. and nicotinic acid, pimelic acid, i-inositol, uracil and p-aminobenzoic acid from the Eastman Kodak Co.

Water extracts of crude casein, egg yolk, yeast (Harris), timothy hay and alfalfa meal (Denver Milling Co.) were also used. Only the last two were used in routine culturing and in experiments on fractionation. The extracts were prepared by boiling 50 g. of material with a liter of water for ten min. and filtering with suction using Celite, analytical grade (Johns-Manville), as a filter aid. The timothy extract was used in a dilution of 1 : 5 and the alfalfa extract in a dilution of 1 : 10.

The crude extracts were treated in various ways in an attempt to remove the protein present as a preliminary to a study of the nitrogen requirements of *T. geleii*. The results of these fractionations were so interesting that studies on them were continued while the work on the nitrogenous nutrition was in progress. Tests for protein or its degradation products were made by the ninhydrin reaction.

One of the first methods tried for the removal of protein was precipitation with lead acetate. A 25 per cent solution of normal lead acetate was added to the extract until precipitation was complete. The precipitate was then filtered off with the aid of suction and Celite. Excess lead was removed from the filtrate and the precipitate was decomposed by the use of phosphate. In the first experiments a 5 per cent solution of phosphoric acid was used but later a saturated solution of trisodium phosphate was found to give better results. The precipitated lead phosphate was then removed by filtration with suction. A similar technique was used in the preparation of the fractions obtained with ferric oxide hydrosol (prepared according to the method of Thomas and Frieden, 1923).

Barium hydroxide was used as a precipitant after the addition of three volumes of alcohol to the extract. This latter step was necessary because barium hydroxide alone caused little precipitation when added to the aqueous extract. In this case barium was removed by the use of sulfuric acid and the alcohol by boiling. When phosphotungstic acid was used the extract was first made acid by the addition of sulfuric acid to give a concentration of 50 per cent. The sulfuric acid alone caused the formation of a precipitate which was removed by filtration before the addition of the phosphotungstic acid. After separation of the phosphotungstic acid precipitate both the filtrate and the precipitate were treated with barium hydroxide to remove the sulfuric acid and the phosphotungstic acid.

The method given by Peters and Van Slyke (1931) for the removal of carbohydrate by the use of copper sulfate and calcium hydroxide was tried and found to remove all the reducing sugars present in the extracts, although protein was not removed. Excess calcium was removed from the filtrate either as the carbonate or the phosphate.

Precipitation was also carried out by the use of alcohol or acetone. After adding sufficient alcohol or acetone to give the desired concentration the extracts were allowed to stand until flocculation was complete. The precipitates were then filtered off. To remove the precipitant the filtrates were boiled or distilled, sometimes under reduced pressure. The precipitates were redissolved in water. The above named solvents as well as ether or acetic acid were also used to make extracts of alfalfa meal, using a Soxhlet apparatus except in the case of the acetic acid. The solvents were removed from the extracts by boiling and the residues taken up in water.

When it was found that the active material in these extracts was adsorbed on charcoal (Norit) and to some extent on Super Filtrol (Filtrol Corp.), attempts were made to obtain elution. Various concentrations of methyl and ethyl alcohols at various pH's were tested. The most successful eluting agent consisted of 50 per cent ethyl alcohol containing 10 per cent ammonium hydroxide. Both of these substances could be removed from the eluates by boiling.

Dialysis of the extracts was carried out in cellophane against distilled water. During the process, which lasted for several days (changing the water outside the cellophane at intervals of 12 hours), a temperature of from 50° to 60° C. was maintained in order to prevent bacterial action. An electric light bulb was used to heat the box in which dialysis was carried out. The diffusate and the dialysate were boiled down or made up to the original volume.

The active materials were tested for stability by adjusting the pH of portions of the extracts to values ranging from pH 3.0 to pH 10.0 and then heating in the autoclave at 15 lbs. pressure for one hour. Extracts were also boiled for 24 hours in the presence of five per cent sulfuric acid in an attempt to remove tannins (Harrison and Roberts, 1939). The results gave an additional test of stability to acid.

In all cases the pH of the extracts or of the fractions was adjusted to approximate neutrality in order to avoid changing the pH of the medium or precipitation of the casein upon addition of the extract.

RESULTS

A. Supplementary Factors

None of the strains of Tetrahymena gave growth in the vitamin-free casein medium alone or with the addition of various known growth factors. Upon the addition of 0.08 per cent of a water extract of yeast (Harris) or a water extract of crude casein good growth was obtained. This demonstrated that the casein was available to the ciliates and that the failure to grow was due to a lack of some substance, although the possibility that some toxic substance was neutralized should be kept in mind.

It was then found that when the basic medium was made up with the inorganic salt solution of Barker and Taylor growth of the ciliates occurred in the third transplant without the addition of any supplement. This growth was, however, extremely slow, taking a week or ten days to reach a maximum density of about 1000 organisms per ml., which was far below that in controls in crude casein or vitamin-free casein supplemented with hay or alfalfa extract. Addition to the basic medium of thiamine, riboflavin, nicotinic acid or pyridoxine either alone or in combination made little or no difference either in the rate or the density of growth. The same may be said of pantothenic acid, p-aminobenzoic acid, uracil, pimelic acid, i-inositol, and biotin methyl ester. These results indicate that some unknown factor (or factors) is required for the maximal growth of Tetrahymena, since crude casein gave far better growth than the basic medium supplemented by any or all of the known compounds mentioned above. The fact that transplantable growth occurs in the unsupplemented vitamin-free casein indicates either that the ciliates are capable of a slow synthesis of all their supplementary requirements or that the casein still contains traces of the required factors. The latter explanation seems more probable in view of the difficulty of obtaining chemically pure proteins. Therefore, until a medium of chemically known composition or one composed entirely of synthetic compounds can be formulated the question of the absolute requirement for various growth promoting substances will have to remain open.

It is clear, however, that for maximal growth the ciliates must be supplied with an outside source of unknown factors. These factors were found to be present in yeast, egg yolk, milk, timothy hay and alfalfa as well as meat (e.g. proteose-peptone). The animal sources were much lower in their content of growth promoting material than the plant sources. The former also contain a much larger proportion of protein material. For these reasons work was chiefly confined to the plant materials, especially since one of the aims was to obtain protein-free extracts of the growth promoting material in order to study the nitrogen requirements of the ciliates.

Preliminary experiments with a water extract of yeast had indicated that treatment with lead acetate gave an active precipitate. The procedure when tested on extracts of hay or alfalfa gave precipitates which were much reduced in activity while the filtrates were usually inactive. A recombination of the two fractions gave growth very nearly equal to that of the controls (Table I). The indications are that some of the preparations may be slightly toxic, but, more important, that there are at least two factors present in hay or alfalfa which are necessary for the maintenance of growth at a maximal rate. For convenience the substance present in the material precipitated by lead acetate will be referred to as factor I and the material present in the filtrate as factor II.

TABLE I

Supplements	0	I	II	A or H
0	0	++	0	++++
I			+++ or ++++	

I—factor I
II—factor II
A—untreated alfalfa extract
H—untreated hay extract.

It was also found that neither factor I nor factor II could be replaced by any one of the known growth supplements nor by a mixture of all the ten tested. This is further evidence for the existence of two substances of unknown structure necessary for maximum growth.

Further purification or a better separation of the two factors was attempted unsuccessfully by precipitation with lead acetate from an alkaline solution. Reprecipitation of the fractions with lead acetate was also unsuccessful, since the products gave evidence of greatly increased toxicity, possibly due to the increased phosphate concentration. In all these preparations protein was found to be present in the filtrate fraction and since both fractions are required for growth, the method is not useful for the removal of protein.

Ferric oxide hydrosol has been used as a protein precipitant. When tested on alfalfa and hay extracts it was found to behave similarly to lead acetate. There was a separation into two fractions, both required for optimum growth. The precipitate contained factor I and the filtrate contained factor II as well as protein (Table II).

The results with hay extract and ferric oxide hydrosol are similar to those given above for alfalfa extract but the separation is not so clear cut. A reprecipitation of the iron hydrosol fractions with lead acetate gave a more complete separation, but there was evidence of an increased toxicity of the fractions.

The material precipitated by sulfuric acid in preparation of the extracts for the addition of phosphotungstic acid was found to be inert whether alone or in the presence of either factor I or factor II. Upon the addition of phosphotungstic acid there was no clear separation into two active fractions and no removal of protein without appreciable loss of activity.

TABLE II

Supplements	0	I	II	FeI	FeII	A
0	0	++	+	±	±	++++
I			+++±		++++	
FeI			+++±		++++	

FeI—iron hydrosol procipitate
FeII—iron hydrosol filtrate
Other symbols as in Table I.

At this point the possibility that carbohydrate might be concerned in the activity of these fractions arose. Since all the reducing sugar in the extracts was found in the filtrate fraction from the lead acetate treatment, this fraction was treated with copper sulfate and calcium hydroxide. A complete removal of the reducing sugars but not of the protein in the preparations was possible without appreciable loss in activity. This treatment may be valuable in the further purification of the factors, since other inert materials appeared to be removed with the sugars.

Since heavy metals failed to remove protein, extractions with various organic solvents was tested as a means of obtaining protein-free preparations. Extracts prepared with ether, acetone, alcohol and acetic acid were found to be inactive or even toxic.

Dialysis also failed to remove protein or protein breakdown products. Some nitrogenous material of this nature was found to be freely diffusible as were both factor I and factor II. The dialysate in all cases was inert; all activity was found in the diffusate. The fact that there was some loss of activity from the extracts during dialysis led to the conclusion that one of the factors is destroyed by light. When the electric light bulb used to heat the box in which dialysis was carried out was screened the loss of activity did not occur. This may be correlated with the progressive loss of activity of extracts exposed to ultraviolet radiation for increasingly longer intervals (Kidder and Dewey, 1942, mistakenly state that factor I is affected by the irradiation). The results indicate that factor II is destroyed by light. This is evidence also for the organic nature of the growth promoting material.

Adsorption upon activated charcoal or Fuller's earth followed by selective elution is a well known means of purification of growth factors. When this method was tested it was found that both factors, as well as protein, are readily adsorbed upon Norit and much less readily upon Super Filtrol. The filtrate after the Norit treatment was completely inert. Both factors (as well as the protein) appear to be eluted by alkaline alcohol (Table III). The elutions from Super Filtrol were more successful, possibly because the materials are less strongly adsorbed. Although this method may be useful in the purification of the separate fractions after precipitation with lead acetate, it was discarded as a means of protein removal.

The tests of the stability of the growth substances to heat at various pH values showed that there was no loss of activity in alfalfa extracts in either acid or alkaline

Table III

Supplements	0	I	II	E	A
0	0	++	0	+++	++++
I			+++	++++	
II				++++	
F	+++	++++	+++±	++++	

F—filtrate after adsorption
E—Eluate from Super Filtrol.

solution. On the other hand proteose-peptone treated at an alkaline pH and used to supplement casein was almost inert. By testing it was found that this was due to a loss of factor I during the treatment (Table IV). Factor I from animal sources therefore appears to be heat-labile. The loss of activity in heat-treated proteose-peptone is not due to the destruction of thiamine. The more drastic treatment such as that described for the removal of tannins destroyed activity entirely, which is further evidence for the organic nature of the supplements.

Table IV

Supplements	0	I	HA	HPP	A
0	0	++	++++	±	++++
I				++++	
II	±	++++		+	
Thiamine	±	++		+	

HA—alfalfa extract heated at high pH
HPP—proteose-peptone heated at high pH.

The last method tested for the removal of protein was precipitation with organic solvents. Both the whole extracts and the fraction (filtrate) containing the protein after lead acetate precipitation were treated by the addition of alcohol up to a concentration of 75 per cent. This method was successful in the removal of protein from the hay extracts but not from the alfalfa extracts. The precipitates obtained from hay were inert and the activity of the filtrates was unaffected (Table V). Whole hay extract treated in this manner was used in the experiments on nitrogen nutrition to be described later.

The effect of the addition of barium hydroxide plus alcohol was tested on the alfalfa extract in the hope of precipitating the protein. It was found, however, that 75 per cent alcohol alone precipitated some of both factors along with some of the protein. The addition of barium then had an effect similar to that of lead acetate in that there was a partial separation of the two factors.

Acetone was next considered as a means of removing protein from alfalfa extracts. Its behavior was similar to that of alcohol in that the active substances were precipitated along with the proteinaceous material, the amount increasing as the concentration of the acetone was increased. At 80 per cent factor I was largely precipitated and factor II to a smaller extent. Since protein-free extracts could be obtained readily from hay, the work on alfalfa was discontinued even though it is a richer source of growth-promoting material.

TABLE V

Supplements	0	I	II	IIp	IIf	H	Hp	Hf
0	0	++	±	±	±	++++	±	++++
I			++++	+±	++++			

IIp—precipitate from alcohol treatment of factor II fraction
IIf—filtrate from the same
Hp—precipitate from alcohol treatment of hay extract
Hf—filtrate from same.

From the above the properties of the two factors may be summarized as follows: soluble in water, moderate concentrations of alcohol and in low concentrations of acetone; insoluble in ether; stable to heat (plant sources only in the case of factor I); dialyzable through cellophane; readily adsorbed on charcoal and less readily upon Super Filtrol; eluted by ammoniated alcohol. Factor II differs from factor I in that the former is not precipitated by the salts of heavy metals and appears to be destroyed by irradiation.

When either Harris gelatin or Eastman de-ashed gelatin was used as a basic medium (1.5 per cent solution) the results obtained were similar to those obtained with casein as a basic medium, except that the population density was smaller. In the Harris gelatin alone slight but transplantable growth, which was somewhat improved upon the addition of inorganic salts, was obtained. The addition of thiamine, riboflavin, pantothenic acid or biotin gave little or no improvement in growth, while the addition of hay extract gave a considerable increase in the rate and density of growth.

With de-ashed gelatin the addition of inorganic salts was necessary and in their presence without the addition of supplements slight transplantable growth occurred. The addition of thiamine or ribroflavin (0.0001 mg. per ml.) or both together gave no improvement in growth. Growth was increased only upon the addition of both hay extract and riboflavin to the medium.

B. Nitrogenous Nutrition

The experiments to be described below are exploratory in nature and have served chiefly to suggest further experiments and modes of attack upon the problem. Some of the work of earlier investigators was repeated in the hope that the use of an adequately supplemented medium might give better results than had been obtained.

The first experiments were carried out upon completely hydrolyzed casein. Such a medium was chosen in the hope of shedding more light upon Lwoff's (1932) hypothesis that polypeptides are required for growth. Acid digestion was used because complete hydrolysis by enzymatic means is difficult if not impossible and alkaline hydrolysis has a destructive effect on many of the amino acids. No growth occurred in the acid hydrolysate even in the presence of what was considered to be adequate supplementation. Attention was then turned to a solution of pure amino acids, also supplemented with protein-free hay extract. This solution was prepared using the ten amino acids found by Rose (1938) to be necessary for the nutrition of the mammal. The amino acids were present in the concentrations found in a one per cent solution of casein. Again no growth occurred in this solution or in various dilutions of it.

Such solutions have an osmotic pressure lower than that of salt solutions readily tolerated by the organism. The explanation for the lack of growth must, therefore, be sought elsewhere. Three other possible explanations for the lack of growth are, *a*) that one (or more) amino acid required for the growth of the organism is lacking, *b*) one or more of the amino acids present is toxic or inhibitory, and *c*) that the organism requires nitrogen in the form of polypeptides.

The possibility of the toxicity of the amino acids was considered first. These experiments were to be correlated with others using gelatin as a basic medium and supplemented with one or more of the amino acids known to be lacking from this protein. For this reason those particular amino acids were added to casein as well as to gelatin in the concentrations in which they are found in a one per cent solution of casein. The results in the two media were strikingly different. With casein it was found that the addition of free amino acids had little or no effect on growth. In the case of gelatin (one per cent vitamin-free gelatin Harris) definite inhibition of growth was found in those cultures containing valine, tyrosine or isoleucine. Hydroxyglutamic acid was not then available. When tryptophane was added to the gelatin there was a large increase in the growth and media containing tryptophane in addition to valine or tyrosine gave better growth than similar media lacking tryptophane. It was found that decreasing the concentration of these amino acids to 0.0025 per cent improved the growth in all cases, although tyrosine, valine and isoleucine still showed inhibition of growth. In all cases the media contained protein-free hay extract.

These experiments were repeated in tube cultures three or four times, but in order to check the observations cultures were incubated in Kidder culture flasks and the growth followed by making counts at intervals of 12 hours. With casein plus 0.01 per cent tyrosine it was found that the population density at the end of the phase of logarithmic growth (48 hours) was 81,000 organisms per ml. and without tyrosine 75,500 per ml. The figures in the case of tryptophane were quite similar, 84,000 and 70,000 respectively, with and without 0.01 per cent tryptophane. The generation times did not differ significantly in any of the media. The differences in population density in these media represent less than one division per ciliate and are not regarded as being of statistical significance.

With one per cent gelatin as a basic medium it was found that the addition of 0.01 per cent tyrosine, valine or isoleucine gave maximum populations of only a few hundred organisms per ml. Gelatin alone gave 15,000 per ml. and with the addition of tryptophane a maximum of 90,000. When the concentration of added amino acid was reduced to 0.0025 per cent tyrosine gave a maximum of 12,000; valine, a maximum of 7,100; isoleucine, a maximum of 900 and tryptophane a maximum of 91,000 organisms per ml. In all cases the generation time was lengthened.

When two per cent gelatin was used a population of 80,000 organisms per ml. was obtained and when 0.002 per cent tryptophane was added the maximum was 230,000 organisms per ml. In this case the amino acid caused no decrease in interdivisional time.

The above results indicate that certain amino acids are detrimental to the growth of Tetrahymena, but suggest that this inhibition is reduced or absent in the presence of large protein molecules such as casein, or in the presence of tryptophane. The growth in two per cent gelatin with and without tryptophane leads plausibility to the theory that large protein molecules or a sufficient concentration of smaller protein molecules in some way decreases the inhibitory effect of free amino acids upon the ciliates. Time did not permit the testing of the more toxic amino acids with the higher concentration of gelatin.

Silk peptone, the only other incomplete protein preparation readily available, gave such good growth when supplemented with hay extract that it was not used as a basic medium for the study of amino acid requirements.

Discussion

Of the four types of substances generally accepted as being required for growth of an organism (inorganic salts, supplementary substances, carbon and nitrogen compounds) it is evident that *Tetrahymena geleii* requires inorganic salts (Hall, 1942; Hall and Cosgrove, 1944 and data presented here), supplementary factors and an organic source of nitrogen which supplies the needs for both carbon and nitrogen. The requirement for a source of carbon separate from the source of nitrogen has never been demonstrated.

At present the question of the supplementary factor requirements of Tetrahymena remains unsettled. So far the claims that thiamine is a growth factor (i.e. an absolute requirement for growth) have not been substantiated. Indeed under certain conditions it is not even to be regarded as a growth stimulant (Kidder and Dewey, 1942). The work of Hall and Cosgrove (1944) fails to refute this claim.

Hall (1942) claims that riboflavin is also a growth factor for Tetrahymena (*Colpidium campylum*). This work could not be confirmed, although stimulation of growth could be obtained with both thiamine and riboflavin under certain conditions. In any case the growth stimulation obtained with the two unknown factors described above is far more powerful than that caused by either of these compounds.

Elliott (1935b) reports an increase in the maximum population density of cultures when pantothenic acid was added to tryptone media. Since he was using a crude preparation of pantothenic acid this effect may have been due to other substances in the preparations. Pantothenic acid has subsequently been found to have have no effect on growth when added to a casein medium. So far as can be determined from the data published (Hall, 1939; 1942) pimelic acid has no "acceleratory" effect on growth. The effect appears to be due to the introduction of inorganic salts.

Certain secondary effects have been attributed to thiamine, riboflavin and other known growth substances (Hall, 1940a; Hall and Shottenfeld, 1941; Baker and

Johnson, 1941). These are concerned with the death and decline phases of growth and are not of immediate interest here. It is of more importance to the problem under consideration that none of the known growth-promoting substances will permit maintenance of growth at the maximum rate and of a maximum density. For such growth at least two substances of unknown nature are required. Whether or not some of the known compounds may also be required for such growth cannot be decided until pure preparations of these substances and a basic medium known to contain no growth supplements are available. The use of purified gelatin for a basic medium may give information of some value, but it is not truly suitable, since its use introduces the complication that it does not satisfy a possible requirement for one or more of the amino acids it lacks. Although the so-called vitamin-free casein is not altogether ideal because it appears to contain traces of growth promoting materials, it is nevertheless an adequate basic medium for a study of growth stimulation. Unsupplemented, the growth it supports is far from maximal.

The fact that growth of these ciliates can be obtained in gelatin solutions when properly supplemented, as pointed out by Hall (1942), would indicate that the ciliate requires for growth none of the amino acids lacking from that protein. In other words *T. geleii* must synthesize tryptophane, valine, hydroxyglutamic acid, isoleucine and possibly tyrosine unless its protoplasm does not contain these amino acids. This latter hypothesis seems most unlikely especially in view of the fact that tryptophane increases the growth so remarkably. It is difficult to explain however, why tryptophane increases the maximum concentration of organisms obtained rather than the growth rate.

The failure of other investigators to obtain growth with solutions of amino acids or incomplete proteins supplemented with amino acids is now understandable. In some cases (Lwoff, 1932; Elliott, 1935b) the media contained none of the supplementary factors now known to have a profound effect on growth. Nor can the claims of Hall and Elliott (1935) regarding the effects of certain amino acids be regarded as conclusive, since their results were expressed as x/x_0. As Kidder (1941b) has pointed out, this method of representation may give an entirely false conception of the results obtained. Another source of possible error in the earlier work may lie in the use of concentrations of amino acids which may now be regarded as inhibitory to growth. It is possible that this difficulty may be overcome by the adsorption of amino acids upon inert colloids and by the use of tryptophane, which appears to decrease the toxicity of other free amino acids.

In view of the inhibitory effect of free amino acids and of the ability of *T. geleii* to grow in an incomplete protein such as gelatin, it is difficult to understand the report of Kline (1943) that *T. geleii* (*Colpidium striatum*) will grow in a solution of 15 amino acids with the addition of various supplements. It is possible that the explanation lies in the fact that different strains of *T. geleii* were used.

No definite decision can as yet be made between the three suggested possibilities for the lack of growth of *T. geleii* in amino acid solutions. The evidence on hand, however, suggests that the factor of toxicity of free amino acids is of some importance. This effect, rather than a requirement for polypeptides, is a possible explanation for the decreasing growth obtained by Lwoff (1932) as the degree of hydrolysis of the medium used was increased. This would be true whether or not adequate supplements were present.

SUMMARY

1. The known growth promoting substances alone or in various combinations are not sufficient for the growth of *Tetrahymena geleii* at a maximal rate and density.

2. At least two unknown substances (factor I and factor II), present in both plant and animal materials, are required for such growth.

3. Factor I is distinguished from factor II by the fact that the former is precipitated by heavy metal salts while the latter is not.

4. Active protein-free preparations of these factors may be prepared from extracts of timothy hay by treatment with ethyl alcohol.

5. Growth of the ciliate could not be obtained in acid digests of casein or in solutions of free amino acids supplemented with the protein-free extract.

6. Tyrosine, valine, and isoleucine were found to be inhibitory in the presence of gelatin, but not in the presence of casein.

7. A large increase in the population density occurred in the presence of tryptophane and gelatin but not with tryptophane and casein.

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THIAMINE AND TETRAHYMENA

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Since the publication of our paper on the biosynthesis of thiamine by *Tetrahymena geleii* and *T. vorax* (Kidder and Dewey, 1942) we have expanded our investigations with regard to the basic medium employed. In the earlier work "vitamin-free casein" (Harris, highest chemical purity) was used exclusively as a base. This protein in a one per cent solution with added salts (modified Osterhout's solution) gave "very little growth" either alone or when any or all of the following vitamins were added: thiamine, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, pimelic acid, i-inositol, uracil and p-aminobenzoic acid. Optimum growth resulted when extracts of plant leaves were added (Dewey, 1941; 1944). Even after the extracts were heated for one or more hours at 123° C. at pH 10–11.5 (to destroy thiamine) it was found that optimum growth again resulted without the addition of thiamine. It was further shown that thiamine had been synthesized by *T. geleii* by testing with *Glaucoma scintillans*, an organism dependent upon exogenous thiamine. A tentative conclusion was reached that there was, in the plant extracts, a factor which in some way made it possible for Tetrahymena to synthesize thiamine. This tentative factor was designated factor S.

It has recently been stated by Hall and Cosgrove (1944) that there was no evidence for the biosynthesis of thiamine by Tetrahymena on the basis that "vitamin-free" casein contains appreciable amounts of thiamine. They were able to obtain transplantable growth, using their strain of *T. geleii* (*Glaucoma piriformis*), in unsupplemented one per cent salted casein but not in heat- and alkali-treated salted casein unless thiamine was added. They believe that our low growth was due to a lack of minerals in the media used. Their criticism regarding the mineral factors is justified, as it was not clearly stated in our paper that salts (modified Osterhout's solution) were added. Inorganic salts were mentioned only in "substances used in the preparation of media."

The following experiments, modified to insure thiamine-free media, confirm our earlier results and again show that *Tetrahymena geleii* (strain W) is entirely independent of an exogenous source of thiamine for indefinitely transplantable growth. Quantitative results are being presented for the first time in this connection.

Material and Methods

Pure culture (bacteria-free) *Tetrahymena geleii* (strain W) was used exclusively in the present study. This is the strain which was used by us on previous occasions (Kidder, 1941a; Dewey, 1941; 1944; Kidder and Dewey, 1942). All experiments were carried out in chemically clean Pyrex tubes or flasks and aseptic technique was employed throughout. All media were prepared with water distilled twice over permanganate in an all-Pyrex still. The following substances were used:

casein (Harris, highest chemical purity); gelatin (Harris, selected grade, refined, vitamin-free); alfalfa leaf meal (Denver Alfalfa Milling and Products Co.); thiamine hydrochloride (Hoffman-LaRoche); riboflavin and l-tryptophane (Merck and Co.); inorganic salts (Baker and Adamson). All media were sterilized by autoclaving.

Base media

1. Heated casein—A two per cent solution (treated as described by Dewey, 1944) of casein was autoclaved for one hour at pH 10. After cooling and neutralizing the concentration was adjusted to one per cent or to 0.5 per cent. This medium is always quite turbid and a precipitate settles out upon standing, so the available casein is considerably reduced from the figures given.

2. Filtered heated casein—Heated casein was allowed to cool, the pH adjusted to 6.8, and the precipitate removed by filtration through a Buchner filter with the aid of Celite (Johns-Manville). The filtrate was then diluted to what would be 0.5 per cent (calculated on the original casein). This gave a light straw colored clear solution which again precipitated slightly upon final sterilization.

3. Casein hydrolysate—Two per cent casein was refluxed 22 hours in a 24 per cent solution of H_2SO_4. The sulphate was removed with $Ba(OH)_2$. The resulting hydrolysate was biuret negative. This hydrolysate was used in a 0.5 per cent concentration (calculated from the original amount of casein used).

4. Gelatin—This was used in a two per cent solution.

5. Heated gelatin—Four per cent gelatin was autoclaved one hour at pH 10. After cooling the pH was adjusted to 6.8 and the solution was diluted to a concentration of two per cent.

6. Gelatin hydrolysate—Four per cent gelatin was refluxed for five hours in a 24 per cent solution of H_2SO_4. The sulphate was removed by $Ba(OH)_2$. This hydrolysate was biuret negative and was used in one per cent concentration (calculated from the original amount of gelatin used).

Alfalfa extract was prepared as described previously (Kidder and Dewey, 1942). After heat and alkali treatment at pH 10 it was adjusted to pH 6.8 and added in a dilution of 1:10 final concentration. This dethiaminized extract is designated A.

Thiamine hydrochloride was added where indicated in the concentration of one microgram per ml. of medium.

To all of the media used in the following experiments were added just before sterilization the following inorganic salts (Hall and Cosgrove, 1944): 0.02 per cent $MgSO_4 \cdot 7H_2O$; 0.02 per cent K_2HPO_4; 0.01 per cent $CaCl_2 \cdot 2H_2O$; 0.00025 per cent $FeCl_2 \cdot 6H_2O$; 0.00001 per cent $MnCl_2 \cdot 4H_2O$; 0.00001 per cent $ZnCl_2$. To all media was also added 0.1 microgram per ml. of riboflavin. Tryptophane was added to the hydrolysed casein (to compensate for loss in hydrolysis) and to all gelatin and gelatin hydrolysates to a concentration of 0.0025 per cent. Experiments with amino acid mixtures now being conducted show that tryptophane is essential to the growth of Tetrahymena. All media were used at pH 6.8–6.9.

A number of preliminary experiments were carried out with each medium in tubes in serial transplants. Each tube contained five ml. of medium. All tube series were inoculated with a bacteriological loop delivering approximately 0.008 ml. Tube series were grown through at least three transplants before any conclusions were drawn, this to eliminate the possibility of carry over of medium from the stock cultures. Tube cultures were incubated at room temperature and transplants were made every 48 hours, except where very slow growth occurred in the early transplants, where longer times were allowed.

The quantitative studies were made using the culture flasks described earlier (Kidder, 1941b). These flasks contained 100 ml. of media. Inoculations were made from third transplant tubes of like media so that the flask cultures represent fourth transplant series. Sterile serological pipettes were used for the inoculations and from 0.1 ml. to 0.5 ml. was added, depending upon the density of the population in the tube from which the inoculation was made. After the first few experiments inoculations were made from cultures within the exponential growth phase and the inoculations were calculated to give an initial count of as near 100 cells per ml. as possible. Flask cultures were incubated at 24.5° C. All flask experiments were repeated at least once.

Our method of counting cells from culture has been described elsewhere (Kidder, 1941b), but it should be noted here that this method gives only viable counts, hence our population counts tend to be lower in the stationary phase and phase of decline than where methods involving the counting of killed cells is employed. These differences are well illustrated in the work of Johnson and Baker (1943).

Generation time (g) was calculated by the use of the formula

$$g = \frac{t \log 2}{\log b - \log a}$$

where $t =$ the time in hours during which the population has been increasing exponentially, $a =$ the number of cells per unit volume at the beginning, and $b =$ the number of cells at the end of time, t.

Experimental

Population Studies

Casein and casein hydrolysate—When a solution of casein is adjusted to pH 10 and autoclaved for one hour to render it thiamine-free, a number of changes take place which make it very inferior to unheated casein as a basic medium for Tetrahymena. Hall and Cosgrove (1944) state that factors in addition to thiamine must have been destroyed, because even upon the addition of thiamine poor growth resulted. With our strain of *T. geleii* heat treated casein plus thiamine (also salts and riboflavin, as mentioned above) inhibited growth even in the first transplant, and second transplants were almost invariably negative. In no case was growth obtained in the third transplant. However, if the insoluble precipitate resulting from such treatment is filtered off and the concentration (originally one per cent before filtration) is reduced by one-half then low but transplantable growth results. The addition of thiamine has no significant effect upon the generation time, length of the logarithmic phase, maximum yield or survival up to the limit of our experiment (Table I; Fig. 1). This indicates that the heat treatment has produced toxic substances which, when reduced in concentration do not inhibit growth entirely. It also shows that Tetrahymena can reproduce without an exogenous source of thiamine.

TABLE I

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days
Heated casein 0.5 per cent + A	5.02	18,000	160,000	61,000
Heated casein 0.5 per cent + A + B_1	4.80	17,500	182,000	82,000
Filtered heated casein	8.35	1,600	5,500	2,000
Filtered heated casein + B_1	8.96	1,800	8,000	3,200
Filtered heated casein +A	4.43	42,000	100,000	57,000
Filtered heated casein + A + B_1	4.27	38,000	110,000	86,000

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts and riboflavin (0.1 micrograms/ml.).

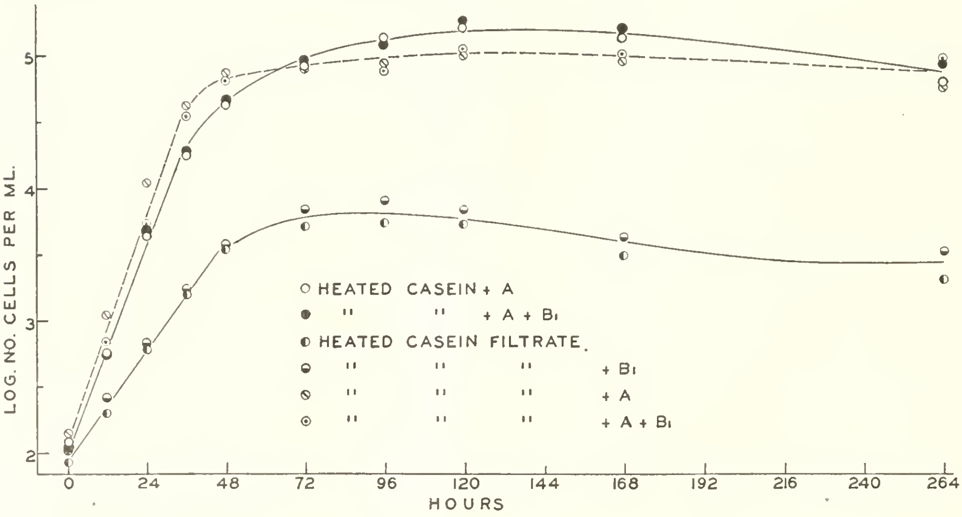

FIGURE 1

When dethiaminized alfalfa extract is added to the heated casein or to the filtered heated casein the response is striking. Rapid growth now occurs in the heated casein while in the filtered heated casein the generation time is reduced by nearly one-half and the population at the end of the logarithmic growth phase is increased from around 1,600 to over 40,000 per ml. The maximum yield is increased from about 5,000 to approximately 100,000 per ml. and a much higher population is maintained for at least 11 days (over 50,000 as compared to 2,000 per ml.) (Table

I; Fig. 1). This would seem to indicate that, in addition to supplying stimulatory factors (Dewey, 1944) and the synthesizing factor, the alfalfa extract counteracts the toxic effects of the heat treatment on the casein. There is indication in the shape of the growth curve that the greater toxicity of the heated casein has not been as successfully counteracted as that of the filtered heated casein. The generation time is approximately 0.5 hour longer in the former and the population begins to fall off sooner. The maximum yield, however, is higher (160,000 as compared to 100,000 per ml.) in the unfiltered casein. This last may be due to the higher concentration of available protein.

Table II

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days
Casein hydrolysate 0.5 per cent + A	4.27	50,000	122,000	49,000
Casein hydrolysate 0.5 per cent + A + B_1	4.36	31,000	191,000	94,000
Heated casein hydrol. 0.5 per cent + A	4.37	29,000	182,000	54,000
Heated casein hydrol. 0.5 per cent + A + B_1	4.46	32,000	171,000	73,000

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

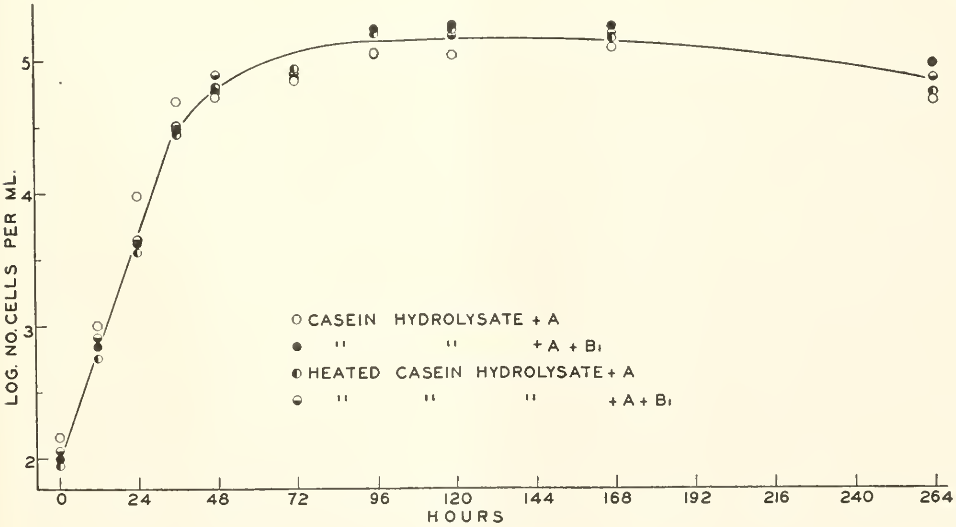

Figure 2

The addition of thiamine to either the heated casein or filtered heated casein plus dethiaminized alfalfa extract has very little effect. The cultures maintain a slightly higher level at 11 days duration but the shape of the growth curves are almost identical.

When a 0.5 per cent solution of a biuret negative casein hydrolysate plus 0.0025 per cent l-tryptophane was used as a basic medium it was found that growth was impossible beyond the first transplant, even when thiamine was added. Inasmuch as unheated casein and filtered heated casein give slow but indefinitely transplantable growth the acid hydrolysis must have destroyed some factor or factors, other than thiamine, necessary for growth. Excellent growth resulted, however, when de-

Table III

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days	Size of cells at 11 days (av. 20 measurements)
Gelatin 2 per cent	5.68	2,600	12,000	2,100	22μ × 16.5μ
Gelatin 2 per cent + B_1	5.59	2,400	67,000	31,000	91.5μ × 34μ
Gelatin 2 per cent + A	3.21	18,000	140,500	47,000	51μ × 24μ
Gelatin 2 per cent + A + B_1	3.22	17,200	161,000	52,000	86.5μ × 22μ

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

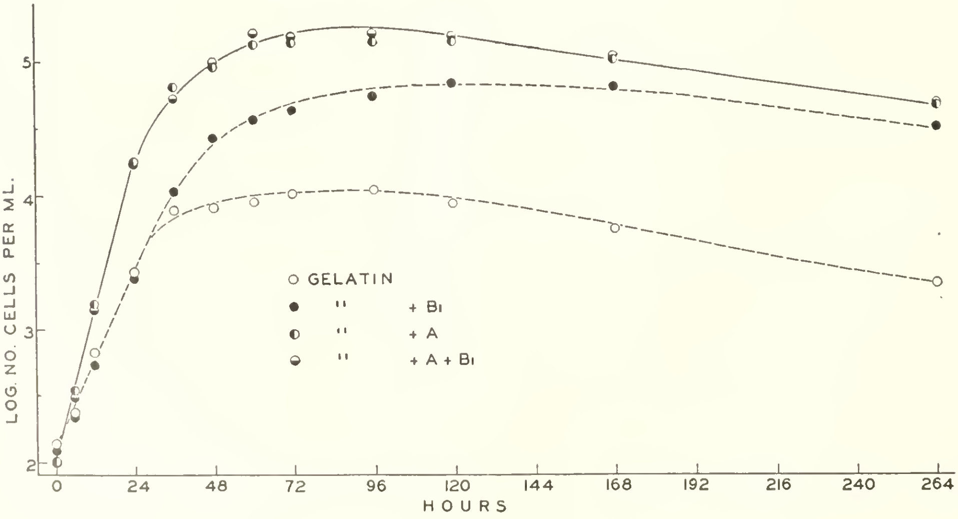

Figure 3

TABLE IV

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days	Size of cells at 11 days (av. 20 measurements)
Heated gelatin 2 per cent	5.31	2,750	11,700	200	20μ × 16μ
Heated gelatin 2 per cent + B_1	5.72	2,000	68,500	44,000	85μ × 30μ
Heated gelatin 2 per cent + A	3.08	19,500	82,000	40,000	47μ × 20μ
Heated gelatin 2 per cent + A + B_1	3.42	17,500	96,000	72,000	89μ × 36.5μ

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

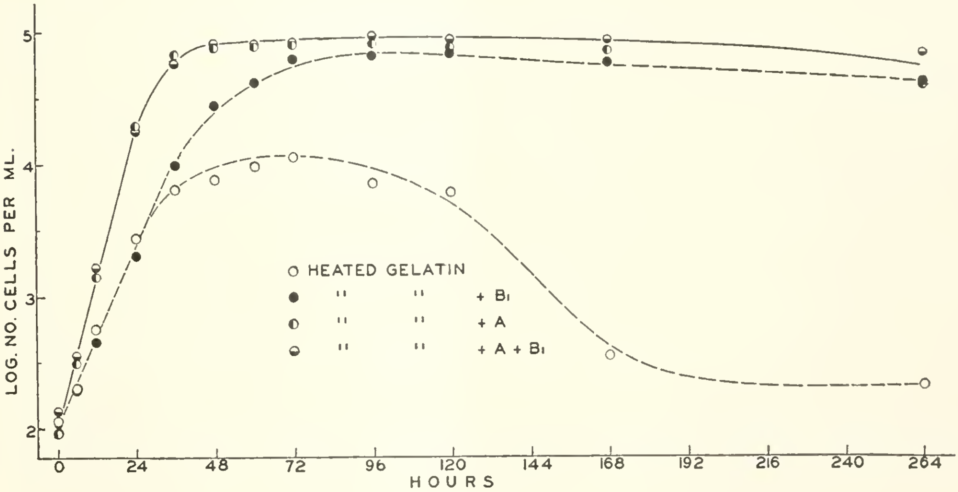

FIGURE 4

thiaminized alfalfa extract was added, and again the addition of thiamine had no effect except upon survival (Table II; Fig. 2).

Gelatin and gelatin hydrolysate—Gelatin Harris is a purified product, according to its manufacturers. It was found, however, that fair growth could be maintained in a one per cent or a two per cent solution provided tryptophane was added. The generation time was about 5.5 hours, the population at the end of logarithmic growth about 2,500, the maximum yield about 12,000 and at the end of 11 days the population was approximately 2,000 per ml. The addition of thiamine had no effect on the generation time or the length of the logarithmic phase but the maximum yield was increased to 67,000 per ml. while at the end of 11 days over 30,000 ciliates per ml. were present (Table III; Fig. 3). This might indicate that enough thiamine was present for limited growth in the unsupplemented gelatin and that added thiamine was necessary for the increased maximum yield and higher survival.

When dethiaminized alfalfa extract is added to gelatin plus tryptophane the generation time is reduced to a little over three hours, the population at the end of logarithmic growth is increased to about 18,000, the maximum yield is increased to over 140,000 and the population at the end of 11 days is increased to 47,000 per ml. (Table III; Fig. 3). This shows the stimulatory effect of the alfalfa factors. The

Table V

Medium	Generation time in hours	Population per ml. at end of log. phase	Maximum yield cells/ml.	Population per ml. at 11 days	Size of cells at 11 days (av. 20 measurements)
Gelatin hydrolysate 1 per cent + A	4.22	57,500	195,000	8,500	50.5μ × 27μ
Gelatin hydrolysate 1 per cent + A + B_1	4.26	51,500	208,000	10,000	99μ × 41μ
Heated gelatin hydrolysate 1 per cent + A	4.59	54,500	160,000	11,000	42.5μ × 19μ
Heated gelatin hydrolysate 1 per cent + A + B_1	4.62	31,000	321,000	9,200	78μ × 35μ

A = heat and alkali treated alfalfa extract; B_1 = thiamine 1 microgram/ml. All media contains salts, riboflavin (0.1 microgram/ml.) and l-tryptophane (0.0025 per cent).

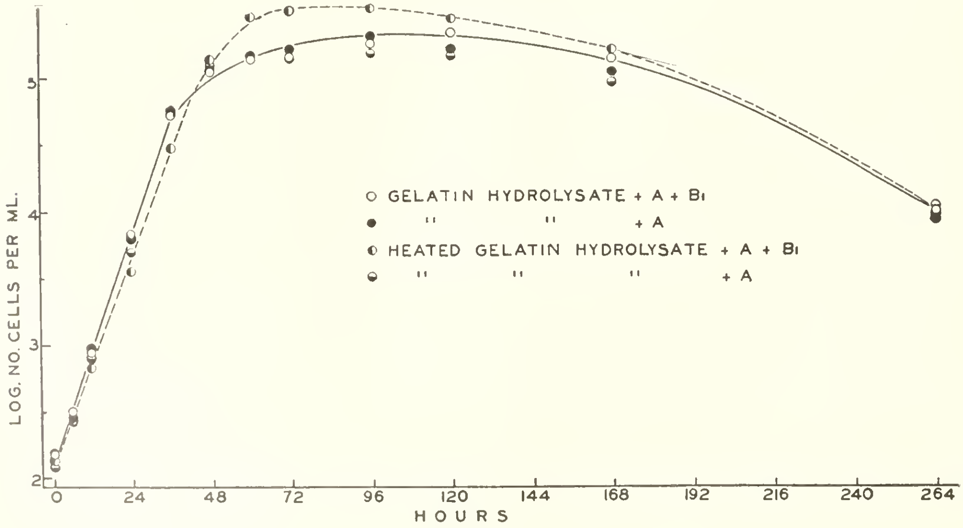

Figure 5

addition of thiamine to gelatin-tryptophane plus dethiaminized alfalfa extract had no significant effect.

When dethiaminized gelatin plus tryptophane is used the only apparent difference from unheated gelatin is in the survival. The ciliate population in heated gelatin begins to decrease more rapidly until at the end of 11 days only about 200 per ml. remain (Table IV; Fig. 4). In eighth transplant tube cultures viable ciliates were found after 30 days. There can be no question that *Tetrahymena geleii* (strain W) does not require an exogenous source of thiamine for limited growth in this medium. Tube cultures were carried through six transplants using glass wool instead of the usual washed and bleached cotton for stoppers, in order to be sure that no trace of thiamine could enter the medium from the few cotton fibers which sometimes drop from the stoppers. All factors required for thiamine synthesis by the ciliates must be present in limited amounts in gelatin and must withstand the rigorous heat and alkali treatment. The addition of thiamine or alfalfa extract or both to heated gelatin produced about the same results as when added to unheated gelatin (Table IV; Fig. 4).

Acid hydrolysis of gelatin destroys factors necessary for the growth of *Tetrahymena geleii* (strain W) for in no case was growth maintained beyond the initial transplants without the alfalfa factors. This is comparable with the casein hydrolysate. The addition of thiamine had no effect.

Excellent growth resulted when dethiaminized alfalfa extract was added to gelatin hydrolysate plus tryptophane. Best growth was obtained in a one per cent solution of the hydrolysate although two per cent gave only slightly lower growth. The addition of thiamine to the hydrolysate plus alfalfa extract had no effect (Table V; Fig. 5).

Heating the gelatin hydrolysate at 123° C. for one hour at pH 10 caused slight changes of doubtful significance. Again excellent growth occurred upon the addition of dethiaminized alfalfa extract and again the addition of thiamine had no effect on population growth (Table V; Fig. 5). This again demonstrates that these ciliates do not require exogenous thiamine for continued growth. In tube cultures the tenth transplant (in heated gelatin hydrolysate plus tryptophane and dethiaminized alfalfa extract) behaves like the first transplant, and with the technique employed there can remain no effective carry over from the stock solution.

Ciliate Size Relations

The above discussion has been concerned only with population growth. The individual cells vary in size and shape to a great extent depending upon the type of medium employed. This variation is never apparent, however, during active multiplication, but only during the stationary phase and the period of population decline. Detailed observations and measurements were made on cultures based on gelatin and gelatin hydrolysate. The differences noted were due to the presence or absence of the alfalfa extract or of thiamine and not to the nitrogen source.

When the ciliates have grown for from 8 to 11 days in a medium lacking both the alfalfa factors and thiamine (outside supply) they become very small (Tables III and IV) and evenly piriform. The protoplasm appears somewhat dense and the motion of the ciliates is very reduced. The small size can be detected with the naked eye in tube cultures. These small ciliates are perfectly viable, however, and normal cultures result upon transplantation into fresh medium.

When thiamine is added to the medium the ciliates increase in size during the stationary period until at the end of 11 days (the time when measurements were taken) they may be as much as 100 μ in length (Tables III and IV). Most of them are flattened and irregular in shape, are fairly active and quite transparent.

When the alfalfa extract is added to the base medium the ciliates in the stationary period and period of decline are about midway in size between those in media with neither alfalfa nor thiamine and those in the media with added thiamine (Table III, IV and V). These ciliates are also flattened and transparent and are actively motile.

When both the alfalfa factors and thiamine are added together large ciliates result. These are about the size and appearance of those in cultures where only thiamine is added (Tables III, IV and V). They are more actively motile, however.

The above observations are preliminary and limited in nature but they indicate differences due to accessory factors and warrant more detailed study.

Discussion

From the results of our experiments with media based on casein and gelatin we can offer the following statements regarding thiamine and *Tetrahymena geleii* (strain W). In a dethiaminized medium of gelatin plus tryptophane or in dethiaminized casein (filtered) plus inorganic salts and riboflavin this ciliate can be serially transplanted apparently indefinitely. It appears to be able to synthesize thiamine enough for moderate to low population growth. This synthetic activity seems to be in direct ratio to the amount of some substance (factor S of Kidder and Dewey, 1942) present in small amounts in the heat-treated casein and gelatin. When thiamine is added to the gelatin medium no effect is shown during the logarithmic phase. But as the factors catalyzing the synthesis of thiamine are being depleted (end of logarithmic phase), reproduction falls off sharply in the gelatin medium alone but continues further for some two and a half divisions when outside thiamine is provided. The presence of toxic products of the heat and alkali treatment on casein makes this medium so unsuitable for the ciliates that growth is limited even with added thiamine.

The raising of the reproductive rate by the addition of "factors I and II" (Dewey, 1944) contained in the alfalfa extract counteracts the toxicity of the heat and alkali treated casein. Also large amounts of "factor S" are made available for the synthesis of thiamine over a longer period of time. Indeed this synthesis is enough to meet the requirements for rapid growth and the addition of an outside supply of thiamine has no effect, until the period of population decline.

It seems evident that the denial by Hall and Cosgrove (1944) of our previous conclusions (that *T. geleii* can synthesize thiamine) can now be dismissed. There can be no question of the thiamine-free nature of our medium. A point of some interest, however, is the fact that they obtained transplantable growth with their strain of Tetrahymena (*Glaucoma piriformis*) in "1 per cent dethiaminized casein" plus salts and thiamine but not when thiamine was omitted. Our strain (W) failed to grow in this strength either with or without thiamine. Strain differences may account for this apparent discrepancy, as we found that *T. geleii* (Hetherington strain), the one used in previous studies in this laboratory (Kidder, 1941b; Kidder and Stuart, 1939; Kidder, Lilly and Claff, 1940), is more resistant to the toxic substance produced by the heat and alkali treatment on casein and grew in serial transplants very slowly and in low concentration in 1 per cent heated casein plus thiamine, riboflavin and salts. Another factor which must be taken into consideration is the fact that Hall and Cosgrove discarded the precipitate after decanting the supernatant fluid from heat and alkali treated 1 per cent casein. This would make their medium similar to our filtered heated casein. In view of the activity of the ciliates in heated gelatin where toxicity is less pronounced we believe the principal effect of thiamine in this case as well as in the case recorded by Hall and Cosgrove was to detoxify the medium. We have similar data with various amino acids in other types of experiments now being carried on. Had Hall and Cosgrove repeated our experiments (1942) by using dethiaminized alfalfa extracts they would not, in all probability, have stated that "the results obtained with Glaucoma piriformis afford no basis for concluding that this ciliate synthesizes thiamin. . . ."

We are in agreement with Hall and Cosgrove (1944) regarding the mineral requirements of *Tetrahymena* and accordingly the inorganic salts were always added. We do not know the specific effect of riboflavin on strain W but knowing that this vitamin is rapidly destroyed in alkaline solution by light it was thought best to add sufficient amounts to insure against its being a limiting factor.

In our previous report (Kidder and Dewey, 1942) we stated that "factor S" had not been detected in material of animal origin. It was pointed out, however, that the production of toxic substances by the heat and alkali treatment might mask the presence of "factor S." We are now of the opinion that small amounts of "factor S" are present in Gelatin Harris and Casein Harris and possibly associated with other animal proteins.

In this study we have assumed the synthesis of thiamine by Tetrahymena on the basis of transplantable growth in completely dethiaminized media. This assumption might be questioned on the basis that there is a possibility that Tetrahymena does not use thiamine in its metabolic activities. As far as we know it would then be unique among plants and animals. It must also be remembered that evidence was presented previously (Kidder and Dewey, 1942) that thiamine was actually synthesized and could be detected by the use of *Glaucoma scintillans,* a thiamine-requiring ciliate. Also in view of the real effects adequate amounts of thiamine have on the population levels and on survival (see Johnson and Baker, 1943) and on size, the inclusion of thiamine in the metabolism of this ciliate is almost certain.

One point of importance which should be discussed here is our success in obtaining growth in completely hydrolysed proteins. Lwoff (1932) found that *"Glaucoma piriformis"* would not grow in abiuretic media. He concluded tentatively that these ciliates require peptides or more complex molecules in addition to growth factors. He did not use anything which would correspond to our alfalfa extract. It seems now that hydrolysis does destroy growth factors which can be re-introduced by the addition of heat-treated alfalfa. The whole problem of the nitrogen requirements is being investigated more thoroughly in this laboratory and will be reported elsewhere, but it can be stated from experiments with amino acids that the theory of the "peptide requirement" of Tetrahymena is no longer tenable. Kline (1943) came to the same conclusion.

On the basis of our experiments we can say that thiamine is no longer to be regarded as a "growth factor", as defined by Lwoff (1936–37), for *Tetrahymena geleii*. In the presence of adequate amounts of a certain substance or substances of unknown chemical nature, which we have called "factor S," thiamine is synthesized in sufficient quantity to insure rapid and heavy growth. We offer the suggestion that the great size differences noted in declining cultures with and without added thiamine is related to an ultimate depletion of "factor S," and hence thiamine, in the completely dethiaminized media.

It is apparent from a comparison of generation times and population densities that gelatin based media are as good or better than those based on casein. This is only true, however, when gelatin is supplemented with tryptophane. In view of the ease with which gelatin can be handled it is to be preferred to casein.

SUMMARY

1. *Tetrahymena geleii* will grow in serial transplants in completely dethiaminized filtered and diluted casein solution plus salts and riboflavin. Added thiamine has no effect upon the population.

2. Growth rate, population at the end of exponential growth and maximum yield are greatly increased when dethiaminized alfalfa extract is added.

3. No growth occurs in completely hydrolysed casein even with added thiamine.

4. Good growth occurs in casein hydrolysate plus dethiaminized alfalfa extract. Heat and alkali treatment of the hydrolysate or the addition of thiamine have no significant effect.

5. Whole gelatin and dethiaminized whole gelatin support transplantable growth. The addition of thiamine has no effect during logarithmic growth but causes an increase in maximum yield.

6. The addition of dethiaminized alfalfa extract to gelatin and to dethiaminized gelatin increases significantly the reproductive rate, population at the end of logarithmic growth and the maximum yield.

7. Gelatin hydrolysate and dethiaminized gelatin hydrolysate support growth only when alfalfa extract is added. The addition of thiamine has no significant effect.

8. Ciliates growing in gelatin or heated gelatin alone become extremely small and sluggish after about the eighth day. They remain viable up to 30 days, however. The addition of thiamine causes the ciliates to become very large in old cultures. The addition of alfalfa extract produces ciliates intermediate in size.

9. The conclusion is reached that casein and gelatin possesses small amounts of "factor S" which makes it possible for *Tetrahymena geleii* to synthesize thiamine. "Factor S" from alfalfa extract, together with "factors I and II" (Dewey, 1944) added to casein or gelatin, produce rapid and heavy growth. Thiamine is not a "growth factor" for *T. geleii* (strain W).

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X-RAYS AND THE REPRODUCTIVE CYCLE IN RING-NECKED PHEASANTS[1]

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While engaged in a study of the effect of visible radiation on the reproductive cycle in pheasants, we found an opportunity to study the effect on the reproductive cycle of x-raying the heads of pheasants.

Normal breeding in pheasants covers the period from the middle of April to about the middle of July in the latitude of Schenectady, New York. After the last eggs are laid, the gonads atrophy and enter a resting period until the next spring when the cycle is repeated. The birds have a single definite reproductive period, are hardy and fairly easily handled, and therefore make desirable experimental material.

In an attempt to secure stimulation of the pituitary gland with acceleration of the reproductive period, the heads of four female pheasants were radiated with 50 and 75 r. of x-rays and of two females with 225 and 425 r. An equal number of males were similarly radiated. The x-ray tube was operated at 200 K.V., the rays being passed through 0.5 mm. copper filter.[2] All other parts of the body were protected by lead-impregnated fabric. The groups receiving 50 and 75 r. were given the total radiation on December 12, 1938, while those receiving the larger doses were given 25 r. on the same date and the remainder on February 1, 1939. Two pairs of pheasants without radiation served as controls. All birds were the same age, of the same strain, and had been raised in the same flock. Each pair of birds was held in a separate pen at the New York State Research Center, Delmar, New York, until two weeks after laying ceased. All were fed the normal breeding ration as used on the State Game Farms.

TABLE I

Dose in roentgens	Egg laying began	Egg laying ceased	Days of laying period	Average eggs per hen	Average eggs per hen per day during laying period
0	Apr. 13	July 2	50	35.5	0.71
50	Apr. 15	May 24	39	13	0.333
75	Apr. 17	May 21	34	11	0.324
225	Apr. 21	May 15	24	8	0.333
425	Apr. 23	May 8	15	6	0.40

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[1] Supported in part by a grant-in-aid from the Society of Sigma Xi.

[2] The radiation was made possible through the cooperation of Dr. Albert Lenz, Schenectady, New York.

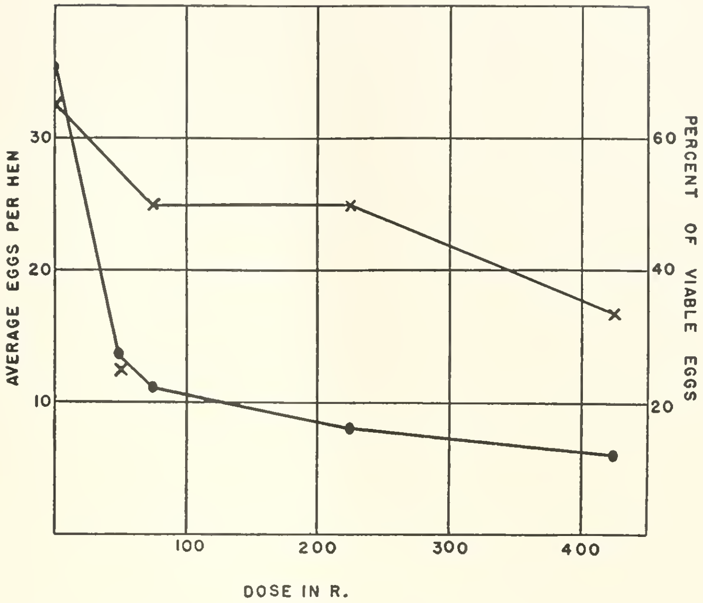

Figure 1. Relation between average eggs per hen and percentage of variable eggs and dose of x-rays. Solid circles, average eggs per hen; crosses, percentage of viable eggs.

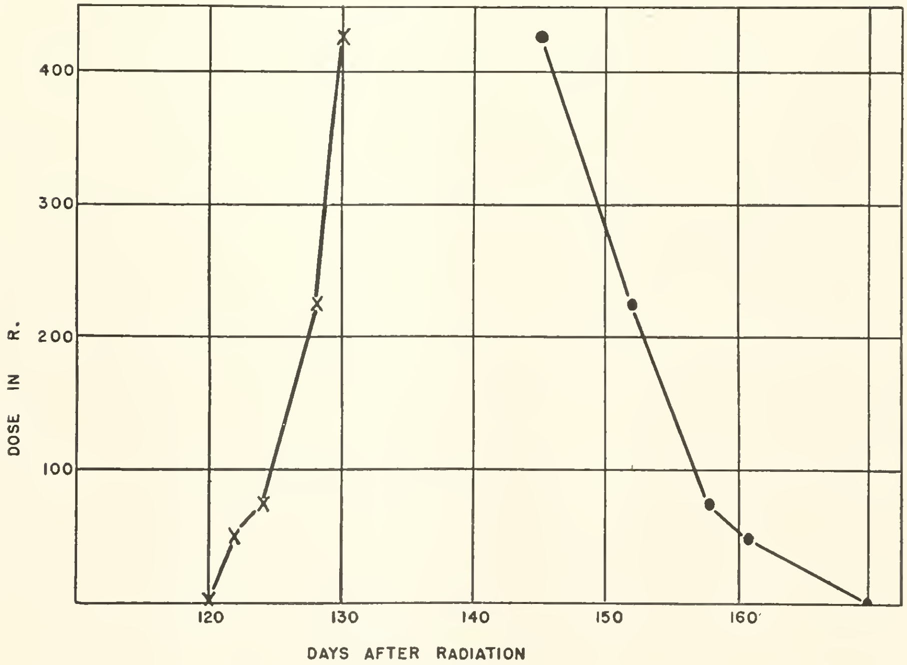

Figure 2. Relation between onset and cessation of egg laying and dose of x-rays.

During the 181 days of the experiment no superficial effects of the radiation appeared. Epidermatitis, loss of feathers or other signs indicating localized effects were absent. The weight of the birds fluctuated, but no more than to be expected in any normal group during the breeding season.

The summary of the effects of radiation on reproduction is given in Table I and Figures 1, 2, and 3.

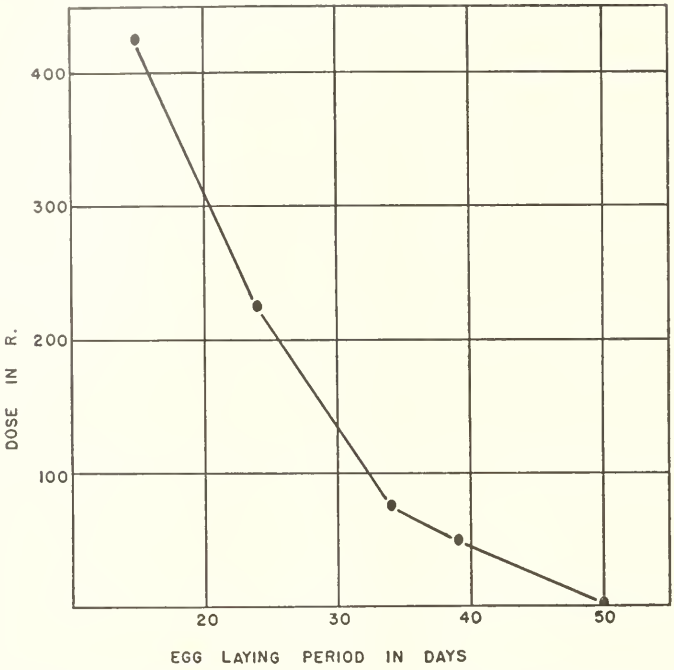

FIGURE 3. Relation between egg laying period and dose of x-rays.

It will be noted (Fig. 1) that the average number of eggs laid per hen varies with the dosage from a high of 35.5 eggs for the controls to 6 eggs for those receiving 425 r.

It will be noted also, Table I and Figure 2, that the time from the beginning of the experiment to the laying of the first egg increases and that the time to cessation of egg laying decreases with the dosage. Thus the laying period varies (Fig 3) from 50 days for the controls to 15 days for birds receiving the maximum radiation. Therefore, the reduced yield of experimental birds must be in part due to their shortened laying period. That this is not the only factor involved is shown in comparing the yield and duration of egg laying of the controls and the birds given 50 r. units of radiation. The ratio of the egg laying periods is 50 : 39 or approximately 1.3 : 1 while the ratio of eggs is 35.5 : 13 or approximately 2.7 : 1. In other words, the controls laid more than twice as many eggs as would be expected if length of laying period were the only factor involved. The rates of egg laying during the active period were calculated and are given in column 6, Table I. It will be seen that rate of laying is essentially the same for all experimental groups, but is less than half that for the control birds.

Although heads only were radiated and the first eggs were laid at least two months later, the viability of the eggs seemed to be affected. A measure of viability was taken as the percentage of eggs pipped or hatched on incubation. It will be seen (Fig. 1, B) that viability decreased from 65 per cent in the controls to 33 per cent in the group receiving 425 r. An exception is found in the group receiving 50 r. where only 15 per cent of eggs were viable, but this low value was due to one hen, all of whose eggs but two were either infertile or had dead germs. Consequently, although the data for that dosage are given it is not considered reliable.

The simplest assumption is that x-rays in the amounts given decreased the amount of pituitary hormone by damaging the cells or causing a prolonged partial inhibition of function. The decrease in egg production, rate of laying, and length of laying period would be manifestations resulting from pituitary disturbance.

SUMMARY

Pheasants given 50, 75, 225 and 425 r. of x-rays applied to the head region show disturbances in their reproductive cycle by decreased egg production, decreased length of laying period, and possibly decreased viability of eggs laid, the amount of decrease being related to the amount of radiation. The rate of egg laying is decreased in the experimental animals but is not correlated with the dose of x-rays over the range studied.

THE EFFECTS OF POTASSIUM CYANIDE, POTASSIUM ARSENITE, AND ETHYL URETHANE ON RESPIRATION IN PELOMYXA CAROLINENSIS[1]

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Introduction

It has been known for some time that cyanide and carbon monoxide inhibit the normal processes of oxidation in living cells. The effect of cyanide on respiration has been studied both on cells of metazoan tissues and on some of the Protozoa. Lund (1918), Gerard and Hyman (1931), and Shoup and Boykin (1931) all found that respiration in Paramecium is not inhibited by cyanide. Lwoff (1934) found that the respiration of *Glaucoma piriformis* in peptone solution was at first reduced as much as 80 per cent by KCN, but later returned to normal or nearly so. Peters (1929) found that M/500 KCN did not inhibit the respiration of *Colpidium colpoda*. Pitts (1932) found that the respiration of *Colpidium campylum* was slightly reduced by cyanide but that this effect was only temporary. Hall (1941), using an improved technique to avoid the loss of cyanide from the test solution, definitely confirmed the fact that *Colpidium campylum* is sensitive to cyanide.

So far as is known, there are no published investigations dealing with the mechanism of respiration in amoeboid organisms. *Pelomyxa carolinensis* Wilson (*Chaos chaos* Schaeffer), a multinucleate rhizopod, is favorable for physiological studies because it is relatively large and can easily be grown in the laboratory.

Material and Methods

The specimens of *Pelomyxa carolinensis* used in these experiments were of the same strain as those used by Belda (1942) and Pace and Belda (1944). They were grown in Hahnert (1932) solution and were fed by adding paramecia to the cultures. Prior to each experiment, however, the pelomyxae were kept for about a week in a culture solution buffered to maintain a hydrogen-ion concentration of pH 6.8 (Pace and Belda, 1944, Table I). Portions of a centrifuged culture of *Paramecium caudatum* were added every second or third day. The pelomyxae grew well under these conditions and usually contained numerous food vacuoles.

The rate of oxygen consumption was measured by means of a Barcroft-Warburg apparatus. Preliminary tests (Pace and Belda, 1944) had shown that there was no measurable difference in the rate of oxygen consumption between pelomyxae tested in flasks which contained 100, 200, or 300 organisms. In the present series of experiments usually 200 specimens were put into each flask; in a few cases 150 or 300 specimens were used.

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[1] With the support of a grant from the American Philosophical Society.

A typical experiment was carried out in the following manner: a 0.4 ml. portion of 10 per cent KOH was put into the inset and a 0.3 ml. portion of 3 N HCl into the onset of 3 of the flasks. These 3 flasks were used as controls. Pelomyxae of uniform size were removed from the buffered culture medium with a capillary pipette under a binocular dissecting microscope and washed in 3 separate portions of fresh sterile culture medium. A 5 ml. portion of sterile culture medium containing the proper number of pelomyxae was then put into each of the 3 Warburg flasks.

A 0.4 ml. portion of a KOH–KCN absorption solution[2] was put into the inset of the 3 remaining flasks and a 0.3 ml. portion of 3 N HCl was put into the onset. Pelomyxae were removed from the buffered culture medium and washed in 3 separate portions of fresh culture medium plus either potassium cyanide, potassium arsenite, or ethyl urethane.

The Barcroft-Warburg apparatus included a total of 7 manometers and flasks. Of these, 6 were prepared as above. A 5 ml. portion of sterile culture solution without pelomyxae was put into the remaining flask which was used as a thermobarometer.

The water bath of the apparatus was kept at 25° ± 0.05° C. The shaking mechanism was operated at the rate of 124 complete cycles per minute through an amplitude of 3 cm. After the manometers and flasks had been put into place with the stopcocks open, the shaking mechanism was run for one hour in order to equalize the temperature of the flasks with that of the water bath. All stopcocks were then closed, and manometer readings were recorded at intervals of one hour.

RESULTS

1. The effect of potassium cyanide on respiration.

In order to ascertain the possible effects of cyanide on the structure and activity of Pelomyxa, several dozen specimens were put into Columbia dishes containing buffered culture solution plus different concentrations of KCN. The specimens were observed carefully under the microscope and compared with other specimens kept in culture solution without KCN.

Practically all the food vacuoles disappear in pelomyxae kept for twelve hours or longer in a solution containing 10^{-2} M KCN. In addition there is a reduction in number or size of both the bipyramidal crystals and the cytoplasmic granules, so that the organisms now appear highly transparent. A number of large vacuoles containing clear fluid are produced in the cytoplasm. Large masses of gelated cytoplasm are found occasionally, both in the interior of the organisms and near the tips of the pseudopodia. Only intermittent movement of the plasmasol can be seen. The hyaline layer appears well-defined, and is much thicker than in normal

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[2] The KCN and KOH concentrations of the absorption solutions suggested by Krebs (1935) vary with the KCN concentration of the experimental culture fluid as shown:

Molar concentration of KCN in culture solution	Absorbing solution in inner cup (inset)
10^{-2}	10 ml. 2N KCN + 0.2 ml. N KOH
10^{-3}	10 ml. N KCN + 1.0 ml. N KOH
10^{-4}	5 ml. N KCN + 5.0 ml. N KOH
10^{-5}	1 ml. N KCN + 10.0 ml. N KOH

specimens. The surface of the pelomyxae is covered with small protuberances.

After 24 hours in the solution, the pelomyxae have long, thread-like pseudopodia. Additional clear vacuoles make their appearance and movement of the cytoplasm practically ceases. The plasmagel layer appears to be very thin and it is difficult to handle the organisms without breaking the outer protoplasmic layers. If the outer layer is ruptured no new membrane is formed in the region of rupture, and the cytoplasm flows out into the surrounding culture medium. In lower concentrations of KCN, namely, 10^{-3}, 10^{-4}, and 10^{-5} M, similar effects occur, but in progressively less degree.

When KCN was added to the buffered culture solution in the higher concentrations used (10^{-2} and 10^{-3} M), the hydrogen-ion concentration was reduced. HCl was added to restore the hydrogen-ion concentration to the value of pH 6.8.

TABLE I

The effect of potassium cyanide on oxygen consumption in *Pelomyxa carolinensis*. Temperature 25° C.; hydrogen-ion concentration, pH 6.8. In most of the tests, 200 pelomyxae were used in each flask; in a few tests, 150 and 300 were used. Average volume of one million pelomyxae, 32,000 cubic millimeters.

Molar concentration of KCN	Number of tests	Duration of tests	Average O_2 consumption in mm.^{3} per hour per million organisms	Average O_2 consumption in mm.^{3} per hour per mm.^{3} cell substance	Per cent inhibition
0 (Control)	8	3 to 6 hours	9045 ± 595	0.282 ± 0.018	65.4
10^{-5}	8		3132 ± 387	0.098 ± 0.012	
0 (Control)	9	3 to 5 hours	8962 ± 641	0.280 ± 0.020	63.1
10^{-4}	9		3220 ± 302	0.100 ± 0.009	
0 (Control)	7	4 to 5 hours	8718 ± 548	0.272 ± 0.017	67.5
10^{-3}	7		2840 ± 345	0.089 ± 0.010	
0 (Control)	8	3 to 4 hours	9478 ± 567	0.296 ± 0.018	69.1
10^{-2}	8		2930 ± 248	0.092 ± 0.008	

The results of the experiments with cyanide are shown in Table I. This table shows that there is a reduction of 63 to 69 per cent in the rate of oxygen consumption in Pelomyxa when KCN is present in the culture solution in concentrations of 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M. The highest concentration of KCN, however, produced only slightly greater inhibition than the lowest. These results indicate that cellular oxidation in Pelomyxa is regulated principally, but not entirely, by the cytochrome-cytochrome oxidase system.

II. The effect of potassium arsenite on respiration

Lwoff (1934) found that respiration in *Glaucoma piriformis* is only slightly reduced, and under certain conditions actually increased in the presence of KCN, and concluded that some respiratory mechanism other than that of cytochrome-cytochrome oxidase must be present in this organism. In an attempt to ascertain whether this mechanism might involve glutathione, he tested specimens of Glaucoma with sodium arsenite. This reagent inhibits the activity of glutathione and other compounds containing –SH groups, but presumably does not affect cytochrome oxidase. Lwoff found that in solutions of 5.26×10^{-4} M and 8.7×10^{-4} M sodium arsenite, respiration in Glaucoma was reduced, respectively, by 75–80 and 90 per cent.

Experiments were carried out with Pelomyxa as above, except that solutions of potassium arsenite instead of potassium cyanide were used. Potassium arsenite was added to buffered culture solution to produce concentrations of 5×10^{-3}, 10^{-3}, 5×10^{-4}, and 10^{-4} M. Additional tests were made with higher concentrations of potassium arsenite, but the organisms were killed in less than 1 hour so that no measurements of changes in the rate of oxygen consumption could be made. The results of the experiments are presented in Table II.

TABLE II

The effect of potassium arsenite on oxygen consumption in *Pelomyxa carolinensis*. Temperature, 25° C.; hydrogen-ion concentration, pH 6.8. Two hundred pelomyxae were used in each flask. Average volume of one million pelomyxae, 34,500 cubic millimeters.

Molar concentration of $KAsO_2$	Number of tests	Duration of tests	Average O_2 consumption in mm.^{3} per hour per million organisms	Average O_2 consumption in mm.^{3} per hour per mm.^{3} cell substance	Per cent inhibition
0 (Control)	6	4 to 5 hours	10,365±467	0.300±0.013	16.3
10^{-4}	7		8,680±525	0.252±0.015	
0 (Control)	6	5 hours	10,240±1,140	0.297±0.033	22.0
5×10^{-4}	6		8,000±467	0.231±0.013	
0 (Control)	7	3 hours	9,894±500	0.287±0.014	29.0
10^{-3}	6		7,025±594	0.203±0.017	
0 (Control)	6	3 to 5 hours	9,831±510	0.285±0.015	34.9
5×10^{-3}	6		6,403±506	0.185±0.015	

This table shows that there is a progressive decrease in oxygen consumption with increasing concentrations of $KAsO_2$. The maximum decrease obtained with the highest concentration of $KAsO_2$ which was not lethal to the pelomyxae was 35 per cent.

III. The effect of ethyl urethane on respiration

Some of the mechanisms of cellular oxidation involve the dehydrogenases. These may be inhibited by the urethanes. Lwoff (1934) found that respiration in *Glaucoma piriformis* was reduced by methyl, ethyl, and propyl urethanes. With 1.66 per cent and 2 per cent ethyl urethane the reduction in oxygen consumption was, respectively, 44 and 57–61 per cent.

Experiments were carried out with *Pelomyxa carolinensis* as above, using ethyl urethane in amounts which yielded concentrations of 0.11 M (1 per cent) and 0.17

M (1.5 per cent) after mixture with culture solution. The results are presented in Table III.

This table shows that with 0.11 M and 0.17 M ethyl urethane the rate of respiration in Pelomyxa decreased, respectively, 35.7 and 65.1 per cent.

TABLE III

The effect of ethyl urethane on oxygen consumption in *Pelomyxa carolinensis*. Temperature, 25° C.; hydrogen-ion concentration, pH 6.8. Two hundred pelomyxae were put into each manometer flask. Average volume of one million organisms, 31,200 cubic millimeters. Duration of each test, 3 hours.

Molar concentration of ethyl urethane	Number of tests	Average O_2 consumption in mm.^{3} per hour per million organisms	Average O_2 consumption in mm.^{3} per hour per mm.^{3} cell substance	Per cent inhibition
0 (Control)	5	10,670±1,240	0.341±0.040	
0.11	6	6,860± 710	0.219±0.023	35.7
0 (Control)	7	9,210±1,100	0.295±0.035	
0.17	6	3,220± 470	0.103±0.015	65.1

DISCUSSION

The results obtained by investigators in earlier tests with cyanide may be erroneous because of rapid loss of HCN from the test solutions. Failure to obtain inhibition of respiration in Paramecium with cyanide has led to the conclusion that the cytochrome-cytochrome oxidase system is not involved in respiration in this organism. However, since Saito and Tamiya (1937) have reported the presence of cytochrome a and c in Paramecium, additional tests, more accurately controlled, should be made.

The fact that Pitts (1932) and Lwoff (1934) obtained only temporary inhibition of respiration in ciliates with KCN, and that Lwoff (1934) obtained inhibition with KCN in peptone solution but not in glucose-Ringer solution, may have been due to loss of cyanide from the test solutions.

The decreased rate of respiration in *Pelomyxa carolinensis* induced by potassium cyanide was maintained as long as the specimens were kept in the solutions, whereas the inhibition brought about by potassium arsenite was only temporary. After eight or nine hours in $KAsO_2$ the rate of respiration had returned nearly to normal; after 15 to 18 hours it was completely normal. These results may indicate that after the supposed inhibition of the glutathione mechanism in Pelomyxa some other respiratory mechanism may begin to function. On the other hand, it may be that there has occurred a gradual conversion of arsenite in the test solution to arsenate.

There is some evidence in the results obtained by Szent-Györgyi and Banga (1933), Korr (1935), and Cohen and Gerard (1937), that arsenite may inhibit the activity not only of glutathione but also of dehydrogenases. If this be true, the results with Pelomyxa indicate that arsenite inhibits the action of dehydrogenases much less than does urethane.

The degree of inhibition of respiration by ethyl urethane in Pelomyxa is approximately equal to that in Glaucoma (Lwoff, 1934), but in approximately equal concentrations of arsenite, respiration in Pelomyxa is inhibited initially by 29 per cent, compared to 75–80 per cent in Glaucoma. If, as supposed, arsenites inhibit the activity of glutathione but not that of respiratory enzymes, it appears that glutathione is much less important in the respiration of Pelomyxa than of Glaucoma.

These results indicate that the respiratory mechanism of *Pelomyxa carolinensis* differs considerably from that of some of the Ciliata. Whether or not the mechanism of respiration of *Pelomyxa carolinensis* resembles that of other free-living Rhizopoda must await further investigation.

SUMMARY

1. In 10^{-5} M KCN respiration in *Pelomyxa carolinensis* is inhibited by 63 per cent. In much higher concentrations of KCN, up to 10^{-2} M, only slightly greater inhibition occurs.

2. Pelomyxae which have been exposed to potassium cyanide (10^{-5} to 10^{-2} M), for 12 to 24 hours, show many changes in protoplasmic structure.

3. In 5×10^{-3} M potassium arsenite the maximum inhibition of respiration in *Pelomyxa carolinensis* is 35 per cent; this effect, however, is only temporary.

4. In 0.17 M (1.5 per cent) ethyl urethane the respiration of *Pelomyxa carolinensis* is inhibited by 65 per cent.

5. Respiration in *Pelomyxa carolinensis* appears to occur chiefly through a cytochrome-cytochrome oxidase system, and partly through a mechanism involving glutathione.

6. The respiratory mechanism of *Pelomyxa carolinensis,* a rhizopod, differs considerably from that of a number of the ciliates.

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THE EXTERNAL MORPHOLOGY OF THE THIRD AND FOURTH ZOEAL STAGES OF THE BLUE CRAB, CALLINECTES SAPIDUS RATHBUN [1]

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For the past two years, workers at the Virginia Fisheries Laboratory, Williamsburg, have been attempting to rear larvae of the commercially important blue crab from the egg through all zoeal stages. In 1941 Dr. Margaret S. Lochhead worked out a successful method of hatching the eggs (Lochhead, Lochhead and Newcombe, 1942) and reared the larvae to the "second zoea" stage. During the summers of 1942 and 1943 this work was continued by Mrs. Mildred Sandoz and Miss Rosalie Rogers, who succeeded in rearing a number of individuals to the "third zoea" stage. The anatomy of the first and second zoeal stages was described in detail by Hopkins (1943). Churchill (1942) described five zoeal stages found in plankton tows at the mouth of Chesapeake Bay. Churchill's first and second zoeae seem to be identical with those reared from blue crab eggs at the Virginia Fisheries Laboratory, but his third zoea is markedly different from the third zoea reared at this laboratory, as reported by Sandoz and Hopkins (1944).

It is now realized, by the agencies concerned with regulation of the crab fishery in Chesapeake Bay, that a more detailed knowledge of the biology of the crab is necessary. Studies of the numbers and seasonal and geographic distribution of larvae in all stages are important means of locating the breeding grounds and determining the length of the larval period, the migrations of the larvae, the percentage of survival under natural conditions, etc. Obviously these studies will be worthless unless the blue crab larvae are correctly identified, and distinguished from the other species of the same family (Portunidae) found in this region. The following paragraphs attempt to give an accurate and detailed description of all features which may be of importance in separating larvae of different species.

A number of individuals in the "fourth zoea" stage have been found in plankton tows made by the Virginia Fisheries Laboratory in the mouth of the Bay near Cape Henry. These are very similar to the blue crab "third zoea" raised in the laboratory and found in plankton, but quite different from Churchill's "fourth zoea" (which was also found in our tows).

The first and second zoeal stages have been restudied, and a few minor corrections of my 1943 description seem necessary. In the first zoea, there are six setae on the endopodite of the first maxilla, two in one group and four in the other. In the second zoea, there are normally three apical setae on the scaphognathite of the second maxilla, although only two can be seen in some specimens. The shorter seta on the fourth segment of the endopodite of the first maxilliped is usually longer than indicated in my 1943 report.

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[1] Joint contribution from the Virginia Fisheries Laboratory of the College of William and Mary and Commission of Fisheries (Number 20): and from the A. and M. College of Texas.

THE THIRD ZOEA

Three specimens reared in the laboratory and six found in plankton tows (Cape Henry, August 14, 1941) were dissected and mounted in glycerine. In addition, a number of specimens from plankton and one specimen reared in the laborator were mounted and studied entire, and still others were studied in formalin without mounting. Even after the most detailed study, no difference could be found between laboratory-reared and plankton specimens, except that one laboratory-reared specimen seemed to have moulted precociously (the telson lacked the fourth or inner pair of setae, the second maxilla was of the second zoea type, and the size was below normal, although the maxillipeds each bore eight swimming hairs).

The total length of the body is difficult to measure accurately because of the bent position of most specimens, but varies between 1.40 and 1.65 mm., measured from front of carapace between eyes to tips of telson. The carapace is exactly as in the second zoea except for larger size and the presence of a single (occasionally two) seta with setules on the posterior edge of the carapace. The dorsal spine is 0.40 to 0.50 mm. long, the lateral spines are 0.09 to 0.10 mm., and the rostrum is 0.33 to 0.36 mm. (measured from lower edges of bases of eyestalks). The eyes have very short stalks and are 0.21 to 0.26 mm. in diameter (Figs. 1 and 2).

The antennule is unchanged from its form in the second zoea except that the setae or aesthetes are less uniform in width; the largest aesthete is about twice as wide as the second, the second is almost twice as wide as the third, and the third is about twice as wide as the fourth; there is also a very short bristle or seta which usually cannot be seen. The peduncle of the antennule is 0.12 to 0.15 mm. long and the length of the longest aesthete is about 0.20 mm. (Fig. 3).

The antenna is 0.30 to 0.35 mm. long. The spinous process bears 12 to 18 hooklike spines on each side. The exopodite is about 0.01 mm. long and bears two setae of unequal length, the longer 0.03 to 0.04 mm. long. There is a very slight ridge or bulge on the antenna near the exopodite which represents the first rudiment of the endopodite.

The labrum bears a distinct chromatophore. The mandible is 0.12 to 0.19 mm. long and 0.08 to 0.11 mm. wide, and bears a very large chromatophore usually divided into three parts.

The maxillule or first maxilla is 0.16 to 0.22 mm. long, from base to end of endopodite. The outer edge of the basipodite bears a seta about 0.04 mm. long. The distal (basal) endite of the protopodite bears eight setae and the proximal (coxal) endite bears six or seven setae. The endopodite is two-segmented; the

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PLATE I

The third zoeal stage of *Callinectes sapidus.* All figures are camera lucida drawings Scale line A represents 0.5 mm. in Figures 1 and 2; scale line B represents 0.2 mm. in Figures 3–7.

FIGURE 1. Third zoea reared from egg in laboratory, slightly flattened under cover glass.
FIGURE 2. Third zoea from plankton tow, Cape Henry.
FIGURE 3. Antennule, third zoea from plankton.
FIGURE 4. First maxilla, third zoea reared in laboratory.
FIGURE 5. Second maxilla, third zoea from plankton.
FIGURE 6. Endopodite of first maxilliped, third zoea reared in laboratory.
FIGURE 7. Endopodite of second maxilliped, third zoea reared in laboratory.

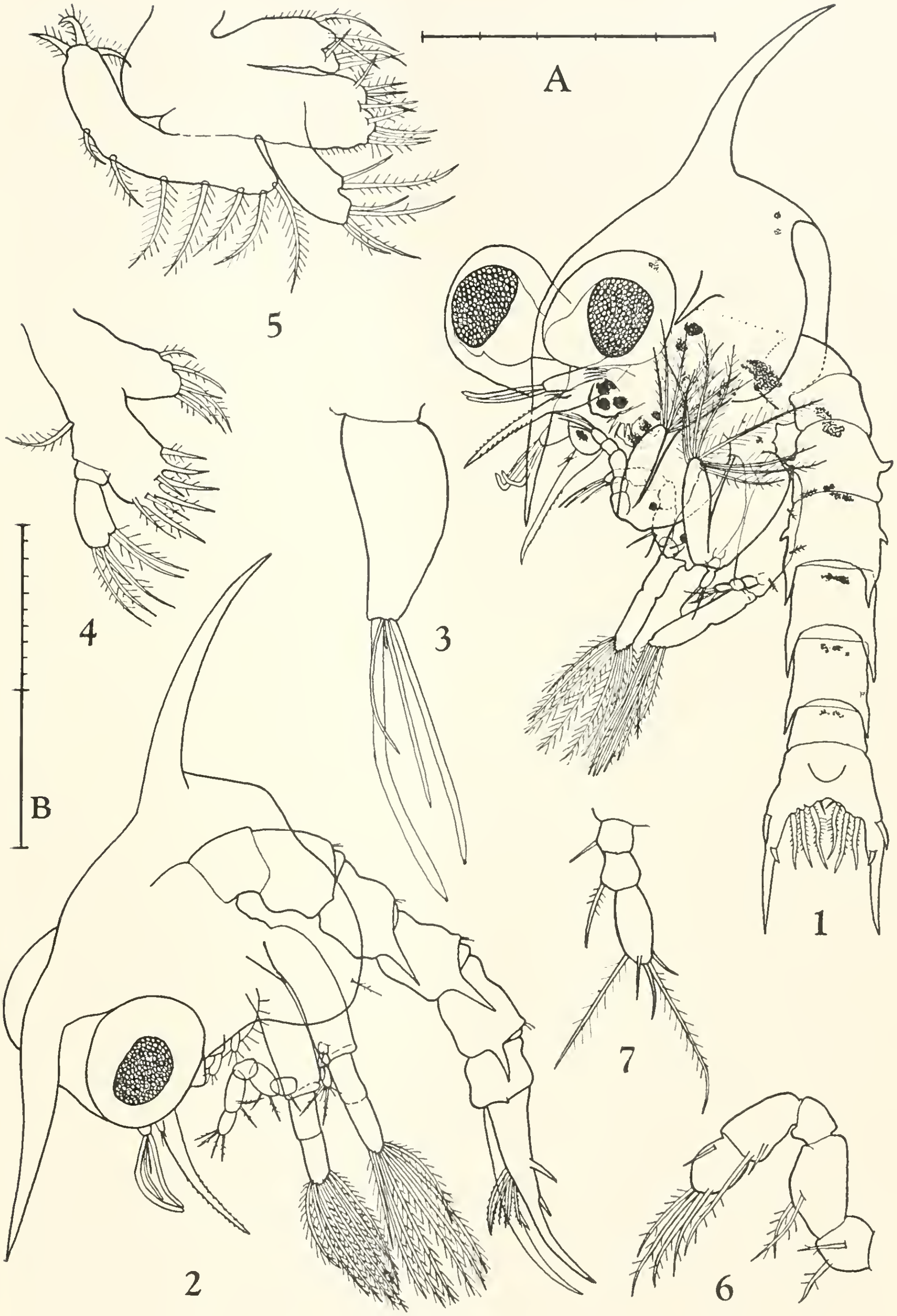

PLATE I

distal segment is bifurcated and bears two groups of setae, four in one group and two in the other. The setae on the basal and coxal endites are 0.03 to 0.06 mm. long and those on the endopodite are 0.04 to 0.08 mm. long; all setae on the first maxilla have setules (Fig. 4).

The second maxilla seems to be subject to considerable variation in size and number of setae. The length of the second maxilla varies from 0.12 to 0.17 mm., measured from the base to the tip of the endopodite. The scaphognathite measures from 0.13 to 0.16 mm. through its longest dimension, and bears seven or eight setae along the distal part of the outer margin plus three to five apical setae around the proximal tip, making a total of ten to twelve setae. The endopodite has a single segment with bilobed tip bearing six setae in two groups, two in one group and four in the other. The basal and coxal endites are both bilobed; the distal (basal) endite bears nine setae and the proximal (coxal) one bears six or seven setae. All setae on the second maxilla bear setules and there are also setules around the margins of the endites (Fig. 5).

The basipodite of the first maxilliped is 0.20 to 0.27 mm. long; its posterior side bears ten setae with setules, and there is a very distinct chromatophore near its distal end. The endopodite is 0.18 to 0.23 mm. long, and has five segments; the first (proximal) segment bears two setae, the next bears two, the third has none, the fourth bears two, and the fifth bears five setae, four terminal and one short lateral seta (Fig. 6). The exopodite is divided into two segments; its length is 0.16 to 0.18 mm.; the eight terminal setae or "swimming hairs" are of unequal lengths, varying from 0.13 to 0.23 mm.

The basipodite of the second maxilliped is 0.20 to 0.24 mm. long and bears four setae with setules near its posterior margin; there is no chromatophore in the basipodite, but there is a rather small chromatophore in the coxopodite. The endopodite is 0.06 to 0.09 mm. long and consists of three segments; the first (proximal) and second segment each bear one seta and the third bears five unequal terminal setae (Fig. 7). The exopodite is 0.18 to 0.23 mm. long, is divided into two segments, and bears eight terminal setae or "swimming hairs" of unequal lengths, 0.15 to 0.27 mm.

The abdomen is 1.05 to 1.20 mm. long and has six segments, not counting the telson. The lateral spines or hooks on the second and third segments are exactly as in the second zoea. The third, fourth, and fifth segments have lateral spines projecting posteriad from the posterior margin of each segment. The second, third, fourth, and fifth segments each have a pair of dorsal setae projecting from the posterior margin, but there is no sign of a dorsal spine on any segment. The first

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PLATE II

The fourth zoeal stage of *Callinectes sapidus*. All figures are camera lucida drawings. Scale line A represents 0.5 mm. in Figure 8; scale line B represents 0.2 mm. in Figures 9–14.

FIGURE 8. Fourth zoea from plankton tow, Cape Henry.
FIGURE 9. Antennule.
FIGURE 10. Antenna.
FIGURE 11. First maxilla.
FIGURE 12. Second maxilla.
FIGURE 13. Endopodite of first maxilliped.
FIGURE 14. Endopodite of second maxilliped.

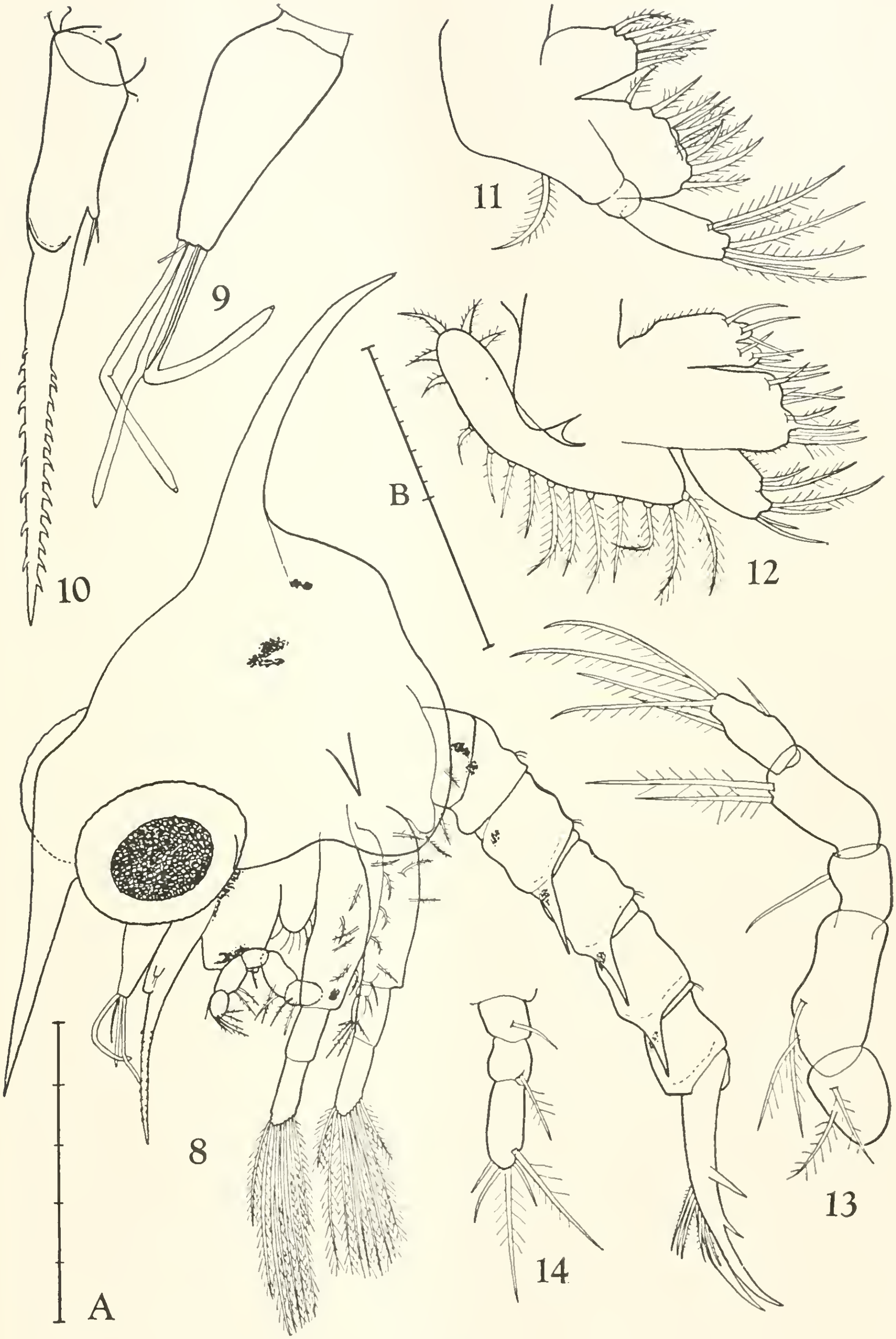
9
11
10
B
12
13
8
14
A

PLATE II

abdominal segment bears a large median chromatophore; each of the other segments bears a chromatophore lying across the extreme anterior end near the ventral surface. Except for slightly larger size, the telson is unchanged from its condition in the second zoea; that is, the dorsal side of each furcus bears a large spine directed dorsolaterally, and a smaller spine, farther back, directed dorsally, and the inner side of each furcus bears close to its base a small spine without setules in addition to the original three setae with setules (Fig. 1).

The chromatophores probably cannot be described accurately without using living specimens, but the following chromatophores have been seen in the best preserved specimens: (1) one chromatophore in the front of the head, between the eyes; (2) one chromatophore dorsal to the anterior part of the alimentary canal; (3) a pair of large chromatophores dorsal to the gut in the posterior part of the cephalothorax; (4) a pair of chromatophores just ventral to the anterior part of the alimentary canal; (5) a small chromatophore, not always visible, below the base of the dorsal carapacial spine; (6) a large chromatophore in the center of the first abdominal segment; (7) chromatophores in the ventral anterior margin of the third, fourth, fifth, and sixth abdominal segments; (8) a large chromatophore in the labrum; (9) a large chromatophore in each mandible; (10) a chromatophore in the distal end of the basipodite of each first maxilliped; (11) a chromatophore in the coxopodite of each second maxilliped.

The Fourth Zoea

Several specimens which are tentatively identified as the "fourth zoea" of the blue crab were found in surface plankton tows near Cape Henry, August 14, 1941. Five of these specimens have been studied in formalin solution, and two have been dissected for more detailed study of appendages. These specimens are identical with the third zoea of the blue crab except for larger size, a better developed endopodite bud on the antenna, additional setae on the maxillae and maxillipeds, and more setae on the posterior edge of the carapace. Identification of this zoea should, of course, be checked by comparison with laboratory-reared fourth zoeae when these can be obtained, but, in the meantime, I have little or no doubt that this is actually the fourth zoea of the blue crab.

The length of the body, from midway between eyes to tips of the telson, is 1.75 to 1.95 mm. The dorsal spine on the carapace is 0.54 to 0.60 mm. long and the lateral spines are about 0.12 mm. long. The length of the rostrum is from 0.45 to 0.50 mm. The eyes are about 0.35 mm. in diameter. The posterior edge of the carapace on each side bears three large setae with setules (Fig. 8).

The antennule is almost exactly as in the third zoea; the peduncle is 0.16 mm. long and the longest aesthete is 0.20 mm. (Fig. 9). The antenna is from 0.35 to 0.42 mm. long and is unchanged from the third zoeal stage except that the bud of the endopodite is now very distinct (Fig. 10); it is noteworthy that the exopodite is still of exactly the same form as in the first zoea. The labrum and mandible are as in the third zoea except for slightly larger size. The maxillule or first maxilla differs from the third zoeal stage only in slightly larger size and in having nine or ten setae on the distal (basal) endite where the third zoea had eight (Fig. 11). The scaphognathite of the second maxilla bears about sixteen setae, with only a slight gap between the setae of the outer margin and the apical setae, which are now similar in form. The basal and coxal endites of the second maxilla are as in the third zoea except that the distal (basal) endite bears nine or ten setae and the proximal (coxal) one has seven (Fig. 12).

The first maxilliped bears eight setae or "swimming hairs" on the exopodite. The endopodite is five-segmented; the first segment bears two setae, the second bears two, the third bears one (lacking in some specimens), the fourth has two, and the terminal segment bears six setae of which four are terminal (Fig. 13). The basipodite of the first maxilliped bears ten setae, located mostly along its posterior edge. The basipodite is 0.28 mm. long, the exopodite is 0.23 to 0.26 mm., and the endopodite is 0.25 to 0.31 mm. The longest seta on the exopodite is 0.32 mm. long. As in previous stages, there is a chromatophore in the distal end of the basipodite (Fig. 8).

The second maxilliped bears ten terminal setae on the exopodite. The endopodite is three-segmented; the first and second segments each bear one seta and the terminal segment has five setae (Fig. 14). The basipodite bears four setae and contains no chromatophore, but the coxopodite does have a chromatophore. The basipodite is 0.30 mm. long, the exopodite 0.24 mm., and the endopodite 0.09 to 0.11 mm. The longest seta on the exopodite is 0.31 to 0.34 mm. long.

The abdomen, including the telson, is identical in every detail with the abdomen of the third zoea, except for larger size.

Discussion

Churchill (1941, 1942) has described five zoeal stages which he assigned to *Callinectes sapidus*. His first zoea and second zoea do belong to this species; they agree with our laboratory-reared zoeae in every detail except that Churchill apparently overlooked the little dorsal spine on each furcus of the telson, which is present in all blue crab zoeae from the first. However, Churchill's third and fourth zoeae, and presumably his fifth zoea also, belong to a different species of crab and have nothing to do with *C. sapidus*. Churchill's third zoea is slightly larger than our laboratory-reared blue crab third zoea, but the most striking differences are the presence of prominent dorsal spines on the fifth abdominal segment (lacking in *C. sapidus*) and the much greater length of the antennal exopodite (which in *C. sapidus* is unchanged from the second zoeal stage). Churchill's third zoea has six swimming hairs on the exopodite of the first maxilliped, and seven on the second, while *C. sapidus* has eight on each maxilliped. There are also minor differences, including the number of setae on the carapace, the degree of development of the appendages following the second maxilliped, and perhaps the numbers of setae on the endopodites of the maxillipeds.

I did not find Churchill's third zoea in my plankton tows, but I did find one specimen of his fourth zoea (in a surface tow off Ocean View, Va., Aug. 14, 1941) and can confirm the accuracy of his excellent figure and description of this stage. Churchill's fourth zoea is much more robust than the fourth zoea which I have assigned to *C. sapidus;* the larger size is obvious to the naked eye. In addition to the possession of dorsal spines on the fifth abdominal segment and the elongated antennal exopodite, his species differs from mine by having seven (instead of three) setae on the posterior edge of the carapace, nine swimming hairs (instead of ten) on the exopodite of the second maxilliped, and greater development of the buds of appendages posterior to the second maxilliped. The distal pair of spines on the furci of the telson are much smaller than the corresponding spines in blue crab zoeae and are on the inner side of the furci rather than the dorsal side as in *C. sapidus*. Both maxillipeds contain chromatophores in the basipodites in Churchill's species, while only the first maxillipeds of *C. sapidus* have chromatophores in the basipodite.

Churchill's description of five zoeal stages which he assigned to *C. sapidus* was based entirely on specimens found in plankton, with the exception of the first zoea. The Virginia Fisheries Laboratory has hatched thousands of blue crab eggs in the laboratory and has reared a few specimens through the second and third zoeal stages, so that the identity of these stages is known beyond question. The identity of the fourth zoea, described and assigned to *C. sapidus* in the present paper, has not been confirmed in this way.

It is certain that Churchill's third zoea does not belong to *C. sapidus;* probably it is a zoeal stage of some other crab of the family Portunidae. The zoeal stages of *Ovalipes ocellatus, Arenaeus cribrarius, Bathynectes superba,* and *Callinectes ornatus* (species which live in or near the mouth of Chesapeake Bay) have never been described, so it is possible that Churchill's species belongs to one of these. It seems probable that his fourth and fifth zoeae belong to the same species as his third zoea, and it is practically certain that they do not belong to *Callinectes sapidus*. The zoeae of several species of *Portunus* described by Lebour (1928) have a long antennal exopodite like Churchill's zoea, but none of them have dorsal spines on the fifth (or any other) abdominal segment.

It seems probable that *C. sapidus* has a fifth zoeal stage which has not yet been seen by anyone.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1944

JULY 18

Melanophore control of the sexual dimorphism of feather pigmentation in the Barred Plymouth Rock. B. H. Willier.

This report deals with the effects of sex-linked genes on the expression of melanophores in the formation of sexual differences in the pigmentation pattern of feathers of the Barred Rock fowl. Melanoblasts from Barred Rock individuals of known sex were introduced into feather germs of the same or of a different breed of fowl, either by grafting them directly into the wing bud of a host embryo or by allowing them to migrate into melanoblast-free wing skin grafts. All possible combinations between melanoblasts of male and female genotypes and the sex of the host and sex genotype of the grafted skin were obtained. A black and white barred pattern was produced in host or graft contour feathers which was invariably in accordance with the sex genotype of the melanoblast introduced. The melanoblasts of the male which are homozygous for two dominant sex-linked genes, barring and silver, and for a dominant autosomal gene, extension, produced a narrow gray-black band and a wide, almost pure white band. Melanoblasts of the female which are homozygous for extension and hemizygous for barring and silver produced a wide black band and a narrow gray-white band. Thus the melanophores of male and female genotypes are provided with different properties for controlling (*a*) the relative width of the light and dark bands, and (*b*) the intensity of their pigmentation. The difference in expression appears to be determined by the number of sex-linked bar genes interacting with the rest of the genotype (mainly with extension and silver). Furthermore, this difference in expression of melanophores of male and female genotypes is manifested independently of sex hormones of the host and of the sex genotype of the feather germs of the grafted skin. It becomes clear, therefore, that sexual dimorphism of barring is a manifestation of differences in expression of male and female melanophores as provided by the number of sex-linked bar genes interacting with the rest of the genotype.

Since melanophores of the same genotype (from same individual), either male or female, produced a barred pattern which varies in quality from feather to feather in the host or grafted skin, it is evident that the individual feather germ has a more or less specific modifying influence on the rhythmic production of pigment by the melanophores. Growth rate appears to be one of the modifying factors since (1) in flight feathers the variations in barred patterns and in growth rate are roughly parallel, and (2) the differences in barred patterns produced in homologous flight feathers of the host and its donor by melanophores of the same genotype appear to be correlated with breed differences in growth rate, being generally higher in the white Leghorn host than in its barred donor. An increase in growth rate is usually correlated with an increase in the relative amount of black pigment in the vane and with a decrease in the distinctness of barring. Other modifying factors acting simultaneously in the feather germ are likewise involved.

The effects of peripheral factors on motor neuron differentiation in the chick embryo. Viktor Hamburger.

If a wing or leg primordium is extripated in a two- or three-day embryo, the spinal ganglia as well as the lateral motor columns of the corresponding parts of the spinal cord become *hypoplastic.* If the central nervous system is overloaded by implantation of a supernumerary limb, a *hyperplastic* effect is observed. The question arises whether the periphery affects the growth of the central nervous system by controlling the mitotic activity or the cellular differentiation. The lateral motor column was selected for an analysis of this problem.

Mitotic counts were made in 20 wing extirpation cases, during the peak of mitotic activity (in most cases on the fifth and sixth day of incubation). A slight depression of the mitotic activity was found on the operated side. However, the observations to be reported presently show that this response is transient and not related to the hypo- or hyperplasia of the lateral motor column.

If cellular proliferation is not affected by wing extirpation or transplantation, then the sum total of all cells of the spinal cord should remain identical on the operated and the unoperated sides of the cord. This expectation was borne out by cell counts of the motor cells, and, separately, non-motor cells in the ventral half of the cord of five older embryos (two cases of wing-bud extirpation, two cases of leg bud extirpation, and one case of wing implantation). In all instances the total cell numbers were strikingly similar on the two sides. In all instances of hypoplasia, moreover, the deficit of large motor neurons was almost exactly compensated by an excess of small non-motor cells; in the case of hyperplasia, a surplus of motor cells was accompanied by a smaller number of non-motor cells. These data give convincing evidence that the proliferative activity of the cord is not permanently impaired by the operation; they leave no doubt but that the peripheral factors control the process of differentiation of small indifferent cells into large motor neurons. An inductive effect is postulated which emanates from a small group of pioneer motor neurons, and which spreads over adjacent indifferent cells, inducing them to differentiate. The newly recruited neurons are added to the lateral motor column and thus increase its inductive capacity. This process of augmentation is not a self-perpetuating mechanism, however, but is under the "remote control" of conditions prevailing at the periphery. It is cut off at the moment when the periphery is saturated with nerve supply. Under experimental conditions, this would happen earlier (hypoplasia) or later (hyperplasia) than under normal conditions.

The superficial gel layer and its role in development. Warren H. Lewis.

Probably every cell and egg and ameboid organism has a superficial gel layer. It exerts continuous contractile tension, a fundamental property of gelled protoplasm. Gel layer and endoplasm are reversible states of the same cytoplasm that readily changes from one state to the other. Local increases and decreases of its contractile tension are responsible for many changes of cell form, extension of nerve axones, cell locomotion, flow of endoplasm, and cleavage of cells and eggs; and during development for the infiltration and interpenetration by migration of individual cells among others of their own type, nerve cells for example, and among others of different types, capillary endothelial spreading, fibroblast infiltrations and myoblast migrations.

The contractile tension of the gel layer over the yolk of the zebra fish egg squeezes endoplasm out of the yolk to form the blastodisc and compresses the yolk globules into polyhedrons. After cleavage this gel layer pulls the blastodisc over the yolk (gastrulation).

Mechanics of invagination. A relative increase of the contractile tensions of the gel layers on one surface of a series of adherent epithelial cells will result in a concave depression (invagination) on the side of the greater tension. Invagination is the resultant of two forces, a distorting one (the contractile tension of the adherent gel layers) and a resisting one (resistance of the cells to distortion). The cells suffer less distortion because of the invagination. In amphibia, an increase in the contraction of the adherent gel layers of the outer surfaces of the blastophore cells is responsible for one phase of blastopore invagination(gastrulation). This contraction also pulls the presumptive endoderm and mesoderm into the walls of the primitive archenteron and the presumptive ectoderm and neural plate towards the blastopore in spite of an opposing contractile tension exerted by the adherent gel layers of the surface cells over the rest of the egg.

The same mechanical principle is involved in the neural tube formation and its subsequent bendings; optic vesicle evagination and invagination to form the optic cup; invaginations of the lens, otic vesicle, nasal pits, and probably in the evaginations of the thyroid, lungs, liver, pancreas, etc.

It also plays a leading and revealing role in the wound healing of eggs.

The contractile tensions exerted by the superficial gel layer of cells thus plays a leading role in early morphogenesis and probably in later stages also, but the role it plays is dependent upon cytoplasmogenesis, cell division, cell growth (increase in protoplasmic mass), cell adhesions, accumulation of intercellular products, etc.

July 25

Ferritin and iron metabolism. L. Michaelis.

A considerable amount of iron is stored in mammalian tissues which is not in the form of any iron-porphyrin compound. 60 years ago Schmiedeberg prepared an iron-containing protein from liver, which he designated as ferratin. It was an ill-defined, not easily reproducible substance. In 1935 Lauffberger discovered that a protein containing as much as over 20 per cent of iron can be obtained as a well-crystallized compound in the form of its cadmium salt from horse's spleen and many other organs of various species. It was designated as ferritin. In 1942, S. Granick and L. Michaelis elaborated a method of separating the iron from the protein. This protein was designated as apoferritin. It resembles the globulins and is not, and does not contain, any nucleoprotein. Whereas ferritin, in spite of its high ability to crystallize, is non-homogeneous on ultracentrifugation, apoferritin is a perfectly homogeneous protein of molecular weight 500,000, as determined by Dr. Rothen. This protein is highly antigenetic. The precipitin reaction shows that it is essentially different from all other known proteins, that it is species-specific, but not organic-specific. Ferritin and apoferritin cannot be distinguished by the precipitin test. Ferritin is brown, apoferritin colorless. In spite of the fact that about 20 per cent of its weight is withdrawn from ferritin, in the form of Fe, on converting it to apoferritin (or about 30 per cent of its weight in the form of ferric hydroxide), the crystal form of apoferritin and ferritin are alike even to such an extent that Dr. Fankuchen could find no essential difference in the X-ray diffraction pattern of the two, except for the fact that diffraction lines are stronger in ferritin than in apoferritin.

The method of preparing ferritin depends on the fact that in an aqueous organ extract, on heating at 80° C. (but not higher) most of the proteins are coagulated, but not ferritin. From the filtrate the remaining protein is salted out by 30 per cent ammonium sulfate, the coagulum redissolved in water and $CdSO_4$ is added. Crystallization proceeds rapidly. The crystals are always isotropic, in the cubic system, either octahedra, often twinned octahedra (horse), or cubes, or tetrahedra in other animals. Human organs yield crystals with slightly curved faces, less regularly shaped. Ferritin can be prepared from many mammals, but not so far from cats or deer, or non-mammalian vertebrates, or invertebrates. It is found in spleen, liver, and bone marrow, and to a small extent (and usually with a smaller iron content, resembling apoferritin), in kidney and testes, but not in blood or muscle. When radioactive iron is injected into anemic dogs, the iron can be retraced after a short time as ferritin in the liver, and under certain circumstances also in the spleen.

The iron of ferritin is always in the ferric state, it is paramagnetic and has a magnetic susceptibility of a characteristic magnitude which does not occur in any other normally occurring iron compound of the organism. It corresponds to a magnetic moment, per gram-atom Fe, of 3.8 Bohr magnetons, which according to theory indicates the presence of three unpaired electrons, whereas in other ferric compounds the number of unpaired electrons in one iron atom is usually either five or one. The ferric hydroxide precipitated from ferritin by NaOH has the same magnetic characteristic.

The facts known so far are scarcely compatible with the assumption that one has to deal with a stoichiometrically well defined iron salt, or iron complex compound, of apoferritin. Very likely, micelles of colloidal iron hydroxide of composition mainly FeOOH, containing small amounts of ferric phosphate in addition, are interspersed in the open spaces of the very loose crystalline structure of apoferritin. Why a special protein is needed for the storage of the iron can not be explained as yet. It is however obvious that iron is accumulated in the form of ferritin as hemoglobin is broken down, and that ferritin furnishes iron for the formation of fresh hemoglobin. It is remarkable that the iron of ferritin is always in the ferric state, that of hemoglobin however in the ferrous state.

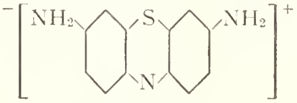
Theory of metachromatic staining. L. Michaelis.

Methods of differential staining, as used in histological technique, may be divided into two classes. The substrate to be stained may be subjected to two (or more) different dyestuffs, either simultaneously or successively, whereby different histological elements absorb selectively the one or the other dye: selective staining. Or, one individual dyestuff may be used to stain various histological elements in different shades of colors: metachromatic staining. Among those dyestuffs exhibiting the metachromatic effect, toluidine blue and thionine are best known. They stain, for instance, all nuclei, or the cytoplasm of lymphocytes, blue; however the same dyes stain the granula of basophilic leucocytes, or mucus, or "amyloid" pathologically occurring in liver and other organs, purple. This difference in color does not depend on pH within wide limits and should be well distinguished from the pH-effect of indicator dyes. All substrates which stain "metachromatically," instead of "normally," are half-esters of sulfuric acid with high-molecular carbohydrates, either linked to a protein or not, e.g. chondroitin sulfuric acid, mucoproteins, and many vegetable colloids among which agar is best known. Metachromatic dyes, therefore, represent a specific chemical reagent that can be used microscopically in situ. Many more dyes exhibit this effect, if the color analysis is carried out spectrophotometrically. It is a more accidental property of the two dyes mentioned above, to reveal the metachromatic effect directly to the unaided dye. On spectrophotometric observation, methylene blue shows this effect at least to the same extent, though it is not very obvious for the unaided eye.

A comparative study of many dyestuffs has revealed that all those dyestuffs stain metachromatically which, in aqueous solution, have the property of forming polymers of the dyestuff molecules in equilibrium with the monomeric dyestuff molecule. Even in rather dilute solution, such dyestuffs form dimeric molecular aggregates, which can be recognized by the fact that the dimers have an absorption band at a wave length different from that of the monomer. Since the percentage of dimerised dye molecules increases with increasing concentration, the molar absorption coefficient, plotted against wave length, varies with the concentration. Such dye stuffs are said to "disobey Beer's law," according to which the molar absorption coefficient should be independent of concentration.

The absorption curve of the metachromatic color, however, is not the same as that of the dimeric dye, rather is the absorption band still more displaced. There is evidence that the metachromatic color is due to high-polymers of the dye. The difference between normal and metachromatic staining, then, consists in the fact, that the surface of "normally staining" substrates adsorbs a monomolecular layer of the dye, and the surface of metachromatically staining "substrate" absorbs a polymolecular layer. All dyestuff molecules are long, flat, almost two-dimensional molecules. Di- and polymerisation consists in piling up these flat molecules plane to plane. The forces which bring about polymerisation are, in a loose sense, comparable to what we may call exaggerated van der Waal's forces. In every case, the metachromatic color turns to the normal color by increasing the temperature, in a reversible way, due to the fact that thermal motion disrupts the aggregates of the dye molecules.

The correlation of the polychromatic effect of a dyestuff with its chemical structure will be discussed in some other place. The following characteristic example may be mentioned. Thionine contains two amino groups at the ends of the elongated molecule, an electric charge oscillating from the one to the other amino group ("resonance"). It is highly metachromatic. Substituting O for S (oxomine) almost abolishes metachromasy. Eliminating one of the two amino groups (monoaminothiazine) destroys metachromasy entirely.



formula of thionin
(univalent cation, as existing in
neutral or slightly acid solution)

The chemical organization of the cytoplasm. Arnold Lazarow.

The organization within the cell permits the coexistence of otherwise incompatible substances such as phosphatase and certain coenzymes. Although the mechanism is little understood, cell organization is determined, in part, by the localization of enzymes within the cell.

The cytoplasm of the liver cell contains several granular components. These may be separated by differential centrifugation after cell fragmentation. The larger particles are the mitochondria. The smaller particles, which are submicroscopic in size, are of two distinct types —one is particulate glycogen, the other is a lipo-protein complex which can be differentiated from the mitochondria by quantitative chemical analysis. Most of the cytoplasmic lipids are concentrated in the mitochondria and submicroscopic lipo-protein particulate. Both contain ribose nucleic acid.

These particulates serve as centers of enzymatic localization within the cell. Since both mitochondria and the submicroscopic lipo-protein particulate oxidize succinic acid, they must contain at least three of the respiratory enzymes—succino dehydrogenase, cytochrome C, and cytochrome oxidase. The mitochondria in addition contain glutamic dehydrogenase. Spatial orientation of the components of the respiratory chain, within the particle, may serve to direct metabolic activity.

The manifestations of a reversible structural framework within the cytoplasm, as evidenced by thixotropy, birefringence (at times), and sol-gel transformations may be explained by the existence of a thread-like micelle similar in type to the tobacco mosaic virus. These elongated micelles can produce a rigid structure even though they are spatially separated by 150Å (a distance several times the diameter of an albumen molecule).

Thus although cytoplasm is organized into particulate components (significant for the localization of some of the respiratory enzymes), micellar components (which may give rise to a reversible structural framework), it nevertheless has a continuous aqueous phase.

August 1

Native protein crystallography and diffraction patterns. Dorothy Wrinch.

Any attempt to understand how native proteins operate in living systems must be based on a knowledge of their atomic organization and of the architectural patterns which characterize them. Unfortunately the instability of these molecules precludes all but the most delicate methods. So far the best and perhaps the only techniques that do not destroy the structure we wish to study are those of physics, particularly X-ray diffraction investigations of protein crystals.

While the protein molecule is not known to be biologically active in crystalline form, a full analysis of insulin crystals (for example) would indicate the nature of the surface pattern of the constituent molecular units. It is confirmed by recent crystallographic work (Fankuchen, *Ann. N. Y. Ac. Sci.* **41**: 157; 1941) that the surface pattern of each protein species, as indicated by the interlinking of units in the crystal, is a highly specific and individualistic tapestry of atoms and electrons. Such molecules can crystallize with vastly different complements of foreign molecules (often water) and for their stability depend on this foreign population. Moreover, irrespective of the foreign element, the protein molecule interlinks with its fellows in characteristic ways and maintains intact its own skeletal structure. In striking contrast to many complex organic crystals, the native proteins in general form crystals of high symmetry.

While these studies have yielded important information about molecular weights and some information about shapes and sizes, they have not as yet uncovered the atomic structure of the native protein particle. To obtain any light on this problem from these studies, it is necessary to interpret the intensity data obtained from X-ray observations. This is a matter of the greatest difficulty.

One line of attack is to study the nature of diffraction patterns in general, a problem of absorbing interest to astronomers for more than a hundred years. Diffraction patterns of apertures of various shapes, repeated in various arrangements, are available for study. It is suggested that the study of such patterns as these may enable us to begin to learn the language of diffraction patterns. A more fundamental attack is also suggested, namely the methodological study of the diffraction patterns of distributions of each and every type. An introduction to such studies by the present writer is in course of publication. The aim is to obtain a clear picture as to how structural features of a known distribution manifest themselves in structural features of its diffraction pattern. This correlation of distributions with their diffraction patterns constitutes an assemblage of mathematical facts essential for the adequate exploitation of the experimental data.

The role of adenylpyrophosphatase in alcoholic fermentation of yeast. Otto Meyerhof.

The Naples Station still lives! Ernst Scharrer.

August 8

On the energy source of the nerve action potential. David Nachmansohn.

In earlier theories acetylcholine (ACh) was supposed to be a "synaptic" transmitter, i.e. a substance released at the nerve ending and acting direcly on a second neuron or on the effector cell. According to the new concept the release and the removal of ACh is an *intracellular* process occurring everywhere at the neuronal surface and directly connected with the nerve action potential. The action of ACh may be pictured in the following way: The nerve is surrounded by a polarized membrane. The polarized state of the membrane is due to a selective permeability to K, which is present in different concentrations on either side of the membrane. During the passage of the impulse the resistance of the membrane is decreased and the permeability to all ions increased. Hereby a local depolarization occurs. This change in permeability appears to be produced by the rapid appearance and removel of ACh. The polarized point becomes negative to the adjacent region and flow of current results. This flow of current stimulates the next following point. There again ACh is released and the whole process repeated. The impulse is thus propagated along the axon. At the nerve ending the surface is increased, the resistance therefore decreased. This leads to a greater flow of current which enables the impulse to cross the non-conducting gap. The transmitting agent is always the electric current, the action potential, but the current is generated by ACh. The picture is consistent with the idea of propagated impulses as developed by Keith Lucas and Adrian. It makes unnecessary to assume that the transmission along axon and across synapses differs fundamentally.

If the release and the removal of ACh are responsible for the alterations of the nerve membrane during the transmission of the nerve impulse, chemical reactions must supply the energy for the resynthesis of ACh. The electric organ of *Electrophorous electricus* offers a suitable material for comparing electrical and chemical changes connected with the action potential since both are in the range of possible measurement.

Such measurements were carried out during the last two years (Nachmansohn, Cox, Coates and Machado). The electric energy released per gram and impulse was found to be eight microcalories, the total electric energy about 48 microcalories. The energy released by phosphocreatine breakdown is about 32 microcalories, that by lactic acid formation 15–18 microcalories. Since the energy of lactic acid formation is probably used, as in muscle, to phosphorylate creatine ("Parnas reaction"), these figures are consistent with the conclusion that phosphate bonds may yield the energy for ACh synthesis.

The amounts of ACh which may be split by one gram of electric tissue during one discharge is about 5×10^{-6} millimole. The amount of phosphocreatine actually split per gram and impulse is about 3×10^{-6} millimole. Thus the amounts of ACh and phosphocreatine metabolized seem to be of the same order of magnitude.

One of the facts supporting the new concept is the extremely high concentration everywhere at the neuronal surface indicating a rate of ACh metabolism sufficiently high to parallel the electric changes. In electric tissue the rate may be at least 100,000 times but probably one million times as high as that of respiration. But we have to distinguish between the possible rate and the absolute amounts metabolized. ACh is released and hydrolyzed within a very short period. The recovery requires one to two hours during which the rate of respiration may be increased. If the absolute amounts are compared, a satisfactory picture is obtained.

A whole chain of reactions connected with the nerve potential could be established. Since it is initiated by the release of ACh, it has been called the "acetylcholine cycle."

As a result of these investigations a new enzyme, choline acetylase, could be extracted from brain which in presence of adenosine triphosphate under strictly anaerobic conditions and in cell-free solution synthesizes ACh.

Current, voltage, and resistance characteristics of injured nerves.[1] Abraham M. Shanes.

Recent studies of the less active aspects of bioelectricity have been almost completely restricted to the "resting" or "injury" potentials. However, if the currents these potential differences produce are also considered, information is obtained which is useful for an understanding of the nature of the potentials and for a study of the permeability of the cell membrane and the conductivity of the protoplasm.

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[1] Aided in part by a grant from the American Academy of Arts and Sciences.

Under the conditions of measurement (injured distal end of nerve in Ringer isotonic KCl solution and ca. 2 cm. of nerve between injured and uninjured regions) currents of 0.2–0.4 microampere are obtained when the injured and uninjured areas of frog sciatic nerve are shorted together. The amount of current drawn can be changed by introducing resistance in the external circuit. Thus it is found that the voltage between the two regions of the nerve varies linearly with this current under all experimental conditions which have been examined. On the basis of Thevenin's theorem it can be concluded that the internal e.m.f. as well as the resistance elements of the nerve fibers are constant up to the largest obtainable currents. This is equally true of the leg nerves of spider and blue crabs, where currents up to 5.5 microamperes are produced.

It is therefore possible to define an internal resistance R as the ratio of the resting potential (i.e., the voltage without external current drain) to the current obtained with a short circuit. In frog nerve this is 100,000 ohms or more, while in invertebrate nerve it measures ⅕–⅒ as great. Preliminary experiments with frog nerve demonstrate that anoxia and excess KCl produce a 4–6 per cent decrease in R, presumably because of changes in the axoplasm, the membrane, or both.

A simple extension of these current and potential measurements makes possible the separate determination of the membrane and protoplasm resistances. Under the conditions of measurement the membrane resistance of frog nerve is 5–10 per cent of the total, while in crab nerve it is insignificant compared with the axoplasm. This difference between the two types of nerves is to be expected from their different structure. The membrane resistance disappears when either is allowed to lower the resting potential to the point of irreversibility. The accuracy of the resistance measurements in the case of frog nerve is indicated by the close agreement of R, measured as described above, with the sum of the separately determined resistances, the agreement usually being within three per cent or better.

Oscillographic studies on the giant nerve fiber system in Lumbricus. Theodore H. Bullock.

This system consists of a median and two lateral fibers on the dorsum of the cord, each comprising a chain of compound axons, separated by septa in each segment. The laterals anastomose frequently. The system mediates the rapid, twitch-like response to startle stimuli and the fibers are said to be polarized, the median conducting backwards, the laterals forwards.

Eccles, Granit and Young (1933) were the first to record the activity of these fibers. This work completely confirms theirs. When the cord is stimulated electrically a consistent pattern of two giant action potential spikes may be recorded anywhere along its length. Above the threshold of these two increase in strength has no effect. The pattern of two agrees with the expectation from anatomy. Further evidence that the giant spikes are related to the giant fibers has been obtained by local thermocoagulation and puncture of single giant fibers. The median fiber has the faster, smaller spike. Speed of conduction of this fiber is of the order of 25–50 m./sec., taken as the elapsed time between stimulus and spike over a gross distance measured on the outside of the animal, i.e. these are minimum figures. They depend on the state of contraction, the rate going up as the animal is stretched, but not proportionally; elapsed time also goes up. The slower fiber conducts at one-third to one-half the rate of the faster. Both are going so fast that there is no significant time available to ascribe to synaptic delay: approximately five milliseconds of elapsed time must be divided between, presumably, about a hundred synapses and about 20 cm. of conduction distance. The pattern of response and the speeds of conduction are the same antero-posteriorly and postero-anteriorly. The two spikes in each direction must be carried by the same fibers. Impulses coming in one direction can block those coming in the other. If simultaneous stimuli are delivered to the cord near the two ends of the animal and the responses are picked up near the middle, two spikes, coming from the nearer stimulating electrodes are recorded, instead of four. The giant fibers, including whatever segmental synapses they have, must be unpolarized. Normal mechanical stimuli at the skin result in the same spikes, but only one spike type from any one site of stimulation: the small, fast spike from a stimulus anywhere in the anterior third of the worm, the large, slow spike from the posterior two-thirds. A sharp line separates the two regions, just behind the clitellum.

Whichever spike is elicited can be detected both in front and behind the site of stimulation, i.e. it is conducted in both directions from this point. The fibers are unpolarized but evidently there are afferent cells connected to the median giant only in the anterior third and connections capable of setting off the lateral giants only in the posterior two thirds. This would explain the apparent polarization found by earlier workers.

Evidence of perpetual proximo-distal growth of nerve fibers.[2] Paul Weiss.

G. H. Parker has postulated a proximo-distal shift of substance in nerve fibers. That some such process really occurs is demonstrated by the following experiments. When a mammalian nerve is chronically constricted by a cuff of artery, the axis cylinders proximal to the constriction assume characteristic shapes, ranging from simple "beading" to ballooning, telescoping, and coiling. These changes (noted in over 50,000 fibers) are most marked immediately at the "bottleneck" and grade off proximally. They appear within a week, and are still present eight months later. They do not perceptibly interfere with nerve function.

The observed configurations resemble closely the damming up of a column of viscous substance driven forward against elastic resistance. One is thus led to the following concept: The neuron, as a living cell, is in a state of constant reconstitution. The synthesis of its protoplasm would be confined to the territory near the nucleus (perikaryon). New substance would constantly be added to the nerve processes from their base. The normal fiber caliber permits unimpeded advance of this mass, with central synthesis and peripheral destruction in balance. Any reduction of caliber impedes proximo-distal progress of the column and thus leads to its damming up, coiling, etc.

This concept is supported by two facts. Firstly, the spacing of the beads (4000 measurements) increases in linear proportion to their distance from the constriction, which is precisely the form to be expected from models. Secondly, release of the constriction after several months is followed by a gradual centrifugal spreading of the dammed up substance with straightening and equalisation of caliber of the affected fibers.

Rate of growth and final caliber of a regenerating nerve fiber vary with the rate of supply from the central cell body. Nerve fibers which have grown through a constricted zone remain small and poorly myelinated in the parts lying distal to the constriction (Weiss and Taylor, *Proc. Soc. Exp. Biol. and Med.*, **55**; 1944).

Our experiments suggest that reproduction of the basic neural protoplasm occurs only near the nucleus. If all cells behave in this manner, this would mean that cytoplasmic reproduction does not occur throughout the cytoplasm, but only in the vicinity of the nucleus, a fact which would have far-reaching implications for our concept of growth.

AUGUST 15

A toxic substance from protoplasm. L. V. Heilbrunn, D. L. Harris, P. G. LeFevre, W. H. Price, W. L. Wilson, and A. A. Woodward, Jr.

The chemical nature of a toxic substance obtained from protoplasm. D. L. Harris, W. H. Price, and L. V. Heilbrunn.

Recent developments in ultraviolet microscopy. George I. Lavin.

In 1904 Köhler (*Zeit. f. wiss. Mikros.*, 21: 129) described a quartz microscope in which the objectives were corrected for the 2750 Å line. The reason for employing such an instrument is that nucleic acid, the proteins, and nucleoproteins have characteristic absorption bands in the ultraviolet region of the spectrum, so it is to be expected that unstained tissue photographed under this condition would show structure not apparent with visible light. Since the resolution is directly dependent on the wavelength of light used, photographs taken with ultraviolet light can be greatly enlarged without undue loss in structure.

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[2] This research was done under a government contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago. It was aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Although work has been done with the original set-up the instrument has been difficult to use owing to the inability of obtaining a satisfactory focus and field. A simplified arrangement has been described (Lavin, *Rev. Sci. Instr.*, **14**: 373; 1943) in which the light source is a resonance mercury vapor lamp (2537 Å), a cobalt sulfate-nickel sulfate filter is used to take out the visible light, and the focussing is carried out with the aid of a willemite screen.

A number of materials such as cancer cells, muscle (Hoagland, Shank and Lavin, *J. Exptl. Med.*, **80**: 9; 1944), nerve cells, marine animal eggs (Harvey and Lavin, *Biol. Bull.*, **86**: 163; 1944) red cells, etc., have been photographed, and the indications are that the instrument will have considerable application since it makes possible the easy study of unstained and in certain cases unfixed tissues.

AUGUST 22

Peripheral circulatory changes during shock produced by hemorrhage. Benjamin W. Zweifach.

The reactions of the capillary bed, representing an organic unit directly concerned with the circulatory failure characteristic of shock, have proved a valuable and sensitive index of the state of the animal throughout the syndrome. Various types of hemorrhage were carried out on dogs of which the omentum had been exteriorized to microscopic observation.

Three types of peripheral circulatory collapse could be produced when hemorrhage alone was the initiating factor. The first is represented by acute hemorrhage, with which no essential dysfunction of the capillary bed occurs. The circulatory collapse is the result of the excessive vasoconstriction of the larger blood vessels and a mechanical failure of the blood to reach the tissues.

The second or intermediate type occurs with graded hemorrhage in which the dog is maintained in a hypotensive state for an extended period and in which there appears progressively a physiologic derangement of the peripheral circulation. This latter condition serves to intensify the general oligemia by the pooling of blood in the capillary vessels and in the small venules.

The third or toxic type was obtained when ether was used as the anesthetic agent, instead of pentobarbital as in the previous experiments. The circulatory collapse involved, in addition to a physiologic derangement, an increase of the normal endothelial permeability, and terminated in true capillary stasis and hemoconcentration.

It is interesting to note the correlation between the degree of dysfunction of the capillary bed and the response to transfusion therapy. The acute hemorrhage type of circulatory failure is responsive to almost any type of fluid infusion, even saline. The second type which involves a progressive derangement of the capillary circulation becomes progressively refractory to crystalloidal fluids, then to colloidal blood substitutes (gelatin, albumin), and finally even to plasma or whole blood. The third type, which possesses the most marked abnormality of the capillary circulation, is least responsive to fluid therapy, over 85 per cent of these animals being refractory to transfusion procedures.

Recent experience with hemoglobin-saline solutions. William R. Amberson, Joye J. Jennings and C. Martin Rhode.

Efforts have been made to prepare and preserve human hemoglobin in a form suitable for intravenous injection into clinical cases. It has not been possible to prepare a dry lyophile product suitable for therapeutic use when redissolved. Such a solution always contains much methemoglobin and some insoluble denatured protein. It is, however, possible to preserve hemoglobin without desiccation or refrigeration by placing sterile solutions in glass vessels which are then rendered oxygen free by evacuation, and sealed. In such solutions methemoglobin formation and denaturation do not occur. In the course of the preparation the stromata must be removed by ether or toluene extraction, and the potassium by dialysis. A pyrogen-free product has been prepared.

Hemoglobin solutions of this type have two possible therapeutic uses. In five human cases of anemia stimulation of hematopoiesis has been observed, after the infusion of 200 to 500 cc. of hemoglobin-saline daily over a number of days. A similar effect has long been known in animals but no human observations have previously been reported. A second use of the solution may be the treatment of certain types of shock. In a single case of shock, caused by massive hemorrhage after childbirth, hemoglobin-saline solutions raised blood pressure and caused return of consciousness after plasma and whole blood infusions had failed to terminate the condition. The patient died with anuria on the eighth day. The case affords hope that such solutions may prove of value in the treatment of shock after hemorrhage, since they are able to transport oxygen as well as to maintain the colloidal osmotic pressure of the blood. Lost blood volume is thereby restored, and the blood pressure returned to normal.

Gelatin as a plasma substitute, with special reference to pseudo-agglutination. Richard G. Abell and William M. Parkins.

That gelatin possesses many of the properties desirable in a plasma substitute has recently been demonstrated by Parkins, Koop, Riegel, Vars and Lockwood (1943). The present investigations on pseudo-agglutination are a part of a larger program being carried out at the University of Pennsylvania by Dr. Parkins and others in which various properties of gelatin in reference to its use as a plasma substitute are being further investigated. The results to be described in this abstract have previously been mentiond briefly in the paper by Parkins et al. referred to above.

It is well known that the addition of gelatin to erythrocytes in vitro causes these cells to adhere in clumps. This phenomenon, known as pseudo-agglutination, has been reported to follow infusions of gelatin by Hanzlik and Karner (1920), Amberson (1937), Stein, Grodins and Dutton (1943) and Grodins (1943). If such clumps are formed within the blood vessels, it is important to know whether they interfere with the blood flow.

Six rabbits and five dogs were infused with six per cent solutions of biological gelatin (courtesy of Dr. D. Tourtellotte, Charles B. Knox Gelatin Co., and Kind and Knox Gelatin Co.) in 0.85 per cent saline, and the effect upon the blood vessels and blood flow observed directly with the microscope in the living animal. In the case of the dogs, the vessels studied were inclosed within intestinal-mesenteric chambers, modified to fit the dog from the original type described by Zintel (1936). Following acute hemorrhage to the point of reducing the blood pressure to 30 mm. Hg, the blood in many of the capillaries and venules became stationary; in the arterioles the flow became sluggish. Replacement of the blood lost by hemorrhage with an equal volume of gelatin caused the blood flow to return to its control rate.

In the normal rapid flow in the dog's mesentery no clumps could be detected following gelatin infusion. Only when the rate of flow was decreased and stagnation induced (by further hemorrhage) could such clumps be seen.

In order to secure further evidence on the manner in which gelatin causes pseudo-agglutination of erythrocytes, transparent moat chambers (Abell, 1932) were inserted in rabbits' ears. After these chambers became vascularized, six per cent solutions of gelatin were injected intravenously (15 cc./kg.) and the effect studied with the microscope.

No pseudo-agglutination could be observed until the rate of flow was reduced by squeezing the main artery of the ear, which supplied the vessels in the chambers. When this was done, it could be seen that erythrocytes that came together side by side adhered to each other to form groups of cells in rouleaux. In control experiments this occurred normally, in the absence of gelatin. Following gelatin infusions, however, the individual rouleaux groups adhered to each other to form larger clumps, made up of several rouleaux groups.

Following gelatin injections, the erythrocytes were seen to pass from the arterioles into the capillaries as separate cells. They did not adhere to each other to form pseudo-agglutinated clumps to any appreciable extent, until they reached the venules. Such clumps did not interfere with the blood flow. When a clump approached a vessel smaller than it was, it separated into its constituent cells, and hence did not block the vessel. The clumps floated in the plasma, which carried them along, and which was always between them and the walls of the vessels. There was no evidence of increase in viscosity of the blood.

Thus, although pseudo-agglutination of erythrocytes does occur following intravenous injections of gelatin, such pseudo-agglutinated cells do not block the vessels or interfere with the blood flow. Consequently, from the standpoint of flow, pseudo-agglutination does not contraindicate the use of gelatin as a plasma substitute.

August 24

Experimental studies on the cytology of allium. C. A. Berger.

The cytological effects of a number of chemical agents were studied in the root tips of *Allium cepa* and compared with the well known effects of colchicine. Acenaphthene, veratrine, sulfanilamide, chloral hydrate and benzene all induced polyploidy and produced cytological effects similar to those of colchicine.

The primary effects of all these substances are the prevention of the formation of an effective spindle and a delay in the division of the spindle attachment regions of the chromosomes. As a result of these primary effects the chromosomes are held at metaphase and become shorter and thicker than normal metaphase chromosomes. After a longer or shorter delay the spindle attachment regions divide, but in the absence of an effective spindle no anaphase movement takes place and the whole group of chromosomes undergoes a revision process giving rise to a tetraploid resting nucleus. After a sufficient period of recovery these cells undergo mitosis as polyploid cells.

Root tips grown in an atmosphere lacking oxygen were found to show similar cytological effects and to produce tetraploid cells. The tentative conclusion is advanced that these effects are not specific to any of the chemicals in question but are general effects common to all the substances in question and interfering with some fundamental metabolic process concerned with the formation of the spindle and the division of the spindle attachment region.

Naphthalene-acetic acid was also used. This substance does not affect the meristem but induces polyploid divisions in the older, differentiated regions of the root. Naphthalene-acetic acid differs from the other substances in that it does not affect cells in division, but causes a double reproduction of the chromosomes in the resting nuclei. At metaphase tetrachromosomes are found. These are four chromosomes held together at a common undivided spindle attachment region. After a slight delay at metaphase two successive divisions of the spindle attachment regions occur. Naphthalene-acetic acid does not inferfere with the formation of the spindle and anaphase separation occurs, resulting in two tetraploid cells.

Studies on the chemical basis of fever. Valy Menkin, M.D.[3]

Fever is usually associated with some form of cell injury. Inflammation is the complex vascular, lymphatic, and tissue response in vertebrates to the presence of an irritant and as such, it represents a manifestation of severe cellular injury. The pattern of injury in inflammation has been shown in earlier studies to be referable to a thermolabile, non-diffusible substance located in the euglobulin fraction of exudates. (*Arch. Path.*, 1943; **36**: 269.) This substance, termed necrosin, appears to be either a proteolytic enzyme or else to have proteolytic activity associated with it. Necrosin, in the form of the toxic euglobulin of exudates, is pyrogenic to both dogs and rabbits. (*Proc. Soc. Exp. Biol. and Med.*, 1943; **54**: 184; *Fed. Proc.*, 1944; **3**: No. 1.) Its formation at the site of injury and its absorption into the circulation offers a reasonable explanation for the basic mechanism of fever accompanying numerous inflammatory processes.

Recent studies indicate that this toxic euglobulin contains a component in turn insoluble in the presence of NaCl or $SO_4^=$. This component is essentially the fever-inducing factor or at least it is associated with that fraction. It can be dissociated from necrosin by treating the exudate with ammonium sulphate at one-third saturation. The precipitate formed is treated with distilled water prior to dialysis of the $SO_4^=$ ions. A true euglobulin enters into the aqueous phase containing the $SO_4^=$ ions. This is necrosin in a further state of purification. It is toxic to mice and is capable of inducing a severe cutaneous inflammation; but it is non-pyrogenic. The pyrogenic factor seems primarily associated with the precipitate which has failed to dissolve in the aqueous phase containing the $SO_4^=$ ions. This highly fever-inducing substance, readily dried by freezing, is termed "*pyrexin.*" Its presence offers a satisfactory explanation for the basic mechanism of fever with inflammation. It is thermostable. Boiling fails to inactivate pyrexin. Ashing destroys it. Incubation of the non-pyrogenic purified necrosin favors the formation of pyrexin. This suggests that pyrexin may be an end product of proteolysis associated with enzymatic activity in the necrosin fraction. It is absent in non-hemolyzed serum, but it is present to some extent in hemolyzed serum and in serum from an animal with a concomitant acute inflammation. It is absent in the pseudoglobulin and albumin fractions of exudates. Pyrexin is excreted, at least in part, in urine. The N and P contents of pyrexin are about ten per cent and one per cent respectively. The material is Biuret negative but Ninhydrin positive, except in the fraction recovered from urine which is usually also Ninhydrin negative. It is Molisch negative. It is insoluble in ether and 95 per cent alcohol, but apparently soluble in relatively weak alkali. The possibility of a peptide attached to a nucleic acid derivative is not precluded by the available data. The exact chemical nature of pyrexin is, however, unknown, and will therefore require further studies. Evidence with barbiturates and antipyretics indicates that the possible mode of action of pyrexin is on the fever centers in the hypothalamic region.

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[3] Fellow of the Guggenheim Research Foundation.

In vitro fertilization and cleavage of human ovarian eggs. John Rock and Miriam F. Menkin.

(This paper has already appeared in *Science*, **100**: 105–107, August 4, 1944.)

AUGUST 29

Phosphoprotein phosphatase, a new enzyme from the frog egg. Daniel L. Harris.[4]

Immediately following homogenization of the ovarian eggs of the leopard frog, *Rana pipiens*, in 0.1 M sodium citrate, there is a rapid liberation of inorganic phosphate from some ester within the eggs. The reaction takes place over a broad pH range in neutral or acid solutions, but there seems to be relatively little hydrolysis in an alkaline medium. There is a pronounced optimum at or near pH 5.0. At pH 5.0 the inorganic phosphate rises from about 23 mg. per cent to 250 mg. per cent in 5 minutes. The speed of the reaction as well as the pH optimum indicates that the hydrolysis is due to an enzyme rather than to the acid conditions. Furthermore, the activity is destroyed by heating.

An analysis of the changes in distribution of phosphate in the brei following "autolysis" as compared with the control in which the reaction was prevented by the addition of trichloracetic acid showed the following: There was no significant change in phospholipid, an increase instead of the expected decrease in organic acid-soluble esters, but a profound decrease in phosphate bound to protein. This decrease in protein phosphate was sufficient to account for the increase in inorganic phosphate as well as the increase in organic acid-soluble phosphate esters. The latter esters have not been identified as yet, but they are known to be resistant compounds, withstanding 1 N HCl for three hours at 100 ° C.

Nucleoproteins and the phosphoproteins of the yolk platelets are the main proteins containing phosphate in the frog egg. To distinguish between these two possible substrates, tests were made using nucleic acid and casein, a typical phosphoprotein, as substrates. There was a rapid liberation of phosphate from casein, but little or none from nucleic acid. Indeed, nucleic acid appeared to inhibit the action of the enzyme on the natural substrate. Vitellin, isolated from the yolk of hen's eggs, was attacked as were yolk platelets which were denatured by heat and added in excess. Sodium β-glycerophosphate and disodium phenylphosphate were hydrolyzed but slowly if at all. The enzyme is, therefore, regarded as a phosphoprotein phosphatase, and the natural substrate within the frog egg is yolk. In the developing embryo the enzyme is thought to liberate inorganic phosphate as needed for carbohydrate metabolism.

The enzyme is bound, in part at least, to the yolk platelets. Lack of contact between the enzyme and substrate can not, therefore, explain the fact that the hydrolytic action of the enzyme is held in check in the ovarian eggs which remain essentially unchanged throughout many months.

Behavior and tube building habits of Polydora ligni. Edith Mortensen and Paul S. Galtsoff.

Polychaete worms of the genus Polydora live on mud bottoms where they cause profound changes by gathering and depositing huge quantities of mud, often covering and smothering other inhabitants with a thick layer of material formed of their loosely constructed mud tubes and excreta. The worms also penetrate between the mantle and the shell of oysters where so-called mud blisters are formed. Thus they sometimes change from a free living to a commensal existence.

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[4] National Research Council Fellow in the Natural Sciences.

In tube building the mud is gathered in a deep ciliated groove extending the length of the inner margin of each of a pair of tentacular cirri as they lash about and secrete a sticky mucus. As the worm turns on its long axis, the mucus-covered mud is passed to the basal end of the tentacles and then is dropped, forming a ring about the anterior end of the body. The mucus is sufficiently adhesive to cause the particles of mud to stick together readily and immediately. There is no systematic placing of the mud in a rotary fashion at the edge of the tube, nor is the material packed together in any way.

That tubes are essential for the survival of *Polydora ligni* was demonstrated in experiments in which two groups of 20 animals were kept under identical conditions with the exception that one group was kept in glass tubing whereas the other was placed free in sea water. On the 23rd day of the experiment, all of the 20 worms left free had perished while 16 of the 20 kept in tubes were still alive.

Rejection of material unsuitable for tube building or food is accomplished by the reversal of ciliary motion along the tentacular groove. When an animal is given substances such as corn starch, Chinese ink, finely ground glass, and sand grains of various sizes, the number of reversals recorded per unit of time is greater than when the same animal is given mud from its natural environment. Likewise the number of reversals occurring when mud soaked in a M/40 KCL solution is used is greater than with mud alone. A 0.01 per cent lactic acid solution added to corn starch gives more reversals than corn starch alone. Thus physical and chemical factors control the reversal of ciliary motion.

The funnel shaped pygidial structure at the posterior end of the body is not a sucker as has been suggested by previous authors but probably a plunger for clearing the tube of excreta.

The conditions which stimulate the entrance of the worms into oysters and cause the free living animal to assume a commensal habit remain unknown. Apparently the worms are not attracted by the oysters and, as laboratory experiments show, may remain in close association with them without penerating their shells. Infestation of oysters by Polydora may be a purely accidental phenomenon.

The click mechanism in elaterid beetles. J. B. Buck.

Several thousand tests on four species of elaterids showed that in click-jumping from an initial dorsum-down position the beetles come to rest on their feet about twice as often as on their dorsal surfaces.

In testing the possibility that this is achieved by controlling the number of aerial loops or twists so that an upright landing results, it was found that: (1) Varying the ratio of height jumped to distance fallen caused no consistent change in the percentage of "successful" (upright) jumps. (2) A tabulation of the direction in which the beetles were facing after jumping indicated that a position in the original plane of the longitudinal axis is favored, and that among the successful jumps those which end with the beetle facing the same direction as originally are somewhat more common than expected. Such a position could only result from a jump including $n + \frac{1}{2}$ twists (rotation on the longitudinal axis) and $n + \frac{1}{2}$ loops (rotation on the transverse axis).

In testing the alternative possibility that the excess of successful jumps is due to body shape or weight distribution, beetles were shaken in a box and dropped or thrown on to a level surface. Beetles dropped in such random fashion land upright about as frequently as after normal jumps, and moreover it makes no difference whether the beetle is dead or alive. Success is enhanced—sometimes to 85 or 90 per cent—by jumping or dropping the beetles on inclined planes, indicating that anything which increases the probability of rolling or bouncing increases their chance of reaching the upright position.

The heights reached in jumping are distributed normally, and the proportion of successful jumps is the same in each height class, so that if success depends on completing a particular number of aerial loops or twists, that number is a constant, independent of the height reached.

Changes in temperature do not affect significantly the proportion of successful jumps.

The evidence summarized indicates that although there may be some path-selection in the mechanism of the jump, the major factor in the preponderance of successful jumps is a predisposition toward the attainment of the upright position, probably due to the external shape of the body.

Metamorphosis in the larva of the Tunicate, Styela partita. L. M. Bertholf and S. O. Mast.

The organisms used in this investigation were kept continuously at 20–21° C.

The average length of larval life (i.e. the time between hatching and the retraction of the tail) varies with the season. It increased from approximately five hours late in June to a maximum of 80 hours early in August and then decreased somewhat. There is great individual variation. It ranged from less than one hour to more than eight days during the course of the summer.

In sea-water in which a few hundred larvae per cc. had previously metamorphosed, the length of larval life is much shorter than it is in normal sea-water. As the number of larvae which had metamorphosed in a given quantity of sea-water increases, the rate of metamorphosis in fresh larvae in this sea-water increases to a maximum and then decreases to zero, i.e. the sea-water becomes so toxic that it kills the larvae before the tail is retracted.

Cupric chloride (2×10^{-6} M.) and Janus green (one part in 250,000 of sea-water), each acting for 3 minutes, greatly accelerate metamorphosis. Neutral red, extract of the muscle of a rabbit killed by x-rays, and concentrated sea-water accelerate it somewhat. Increases in hydrogen-ion concentration from pH 8.05 to pH 7.47, dilution of sea-water up to 40 per cent, crowding of the larvae (1000 per cc. of sea-water) have no measurable effect.

We postulate the following hypothesis of metamorphosis in this animal: It is known that the larva consists of organized adult tissue and organized larval tissue. The larval tissue probably produces a substance which in low concentration augments metabolism and in high concentrations retards it. This substance, then, would be more concentrated in the larval tissue, where it is produced, than in the adult tissue, into which it diffuses. It therefore, at a certain concentration, would retard metabolism in the former and augment it in the latter to such an extent that metamorphosis is initiated.

This substance doubtless diffuses out of the larval tissue into the surrounding medium. Sea-water in which metamorphosis has occurred, therefore, contains some of this substance, which diffuses into fresh larvae put into it, and consequently increases the substance in the larval tissue and thus accelerates metamorphosis. Cupric chloride, Janus green, and other compounds which accelerate metamorphosis probably merely increase the retarding effect of the substance produced by the larval tissue.

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A PRIMITIVE COCCID CHROMOSOME CYCLE IN PUTO SP.

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INTRODUCTION

Every coccid thus far studied presents such striking peculiarities in its meiosis that we are confronted with the paradox of regarding a simple and orthodox maturation process as of especial interest. *Puto* sp. of the family Pseudococcidae reveals a primitive chromosome cycle possibly archetypal for coccids. Only in the llaveiine tribe of the family Margarodidae have partially comparable conditions been encountered. Thus in *Llaveia bouvari* we find as probably primitive traits a sex ratio which approaches equality, no trace of parthenogenesis nor of hermaphroditism, and an XX - XO sex chromosome mechanism. But even in this relatively generalized species a highly specialized achromatic figure has been evolved in male meiosis, and asynapsis of one pair of autosomes is already established as a constant and normal feature in a certain percentage of the spermatocytes. Moreover, in Llaveia the secondary pairing of homologous chromosomes just prior to the second meiotic division provides a mechanism which ensures segregation without previous synapsis—an essential preliminary step to the successful operation of the completely asynaptic habit as encountered in the related genus Protortonia. Puto, while it shares with Llaveia the primitive traits listed above, shows none of the specializations just enumerated. A survey of its cytology discloses a primitive, typically hemipteran pattern and further permits the recognition of certain phenomena as basic coccid characteristics independent of the specialized modifications encountered in the different groups.

MATERIAL AND METHODS

Specimens of this coccid have been deposited with Dr. Harold Morrison of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, Washington, D. C., to whom grateful acknowledgment is made for his assistance. Dr. Morrison reports that the genus Puto is in so confused a state taxonomically as to preclude a specific identification at the present time.

The material was collected near the village of Tequisistlan, Oaxaca, Mexico, in November 1933. All instars were represented at this time, as again in more sporadic infestations found near Tehuantepec, Oaxaca, in December, 1938. The favorite host plant was the stinging-haired *Jatropha* known locally as the "mala mujer."

Male nymphs of the third and fourth instars and adult females with eggs and embryos were dissected in Allen's Bouin. This fixative gave good results in embryonic and late meiotic stages but proved unsuitable for early meiosis. Male material was sectioned at four and female at six micra, and stained in Iron Hematoxylin.

Chromosome Complement

The chromosomes of the female Puto are 14 in number and comprise 7 pairs differing slightly in length (Fig. 1). The male diploid set numbers 13, of which the next to shortest element is the unpaired sex chromosome (Fig. 2).

Somatic Mitosis

Somatic mitosis conforms to the hemipteran type. Its most characteristic features derive from the possession by the chromosomes of a diffuse, in contrast to a localized, kinetochore. Thus the whole body of the chromosome orients at metaphase, chromosomal fibers form from the poleward surface of each chromatid along its entire length, and anaphasic disjunction is parallel (Figs. 3, 4, and 5). In Puto the chromosomal fibers converge to division centers in which a minute centriole may often be discerned. Neither astral rays nor continuous fibers are present. The association between the constituent chromatids of the chromosome is closer throughout the mitotic cycle than in most coccids. (This effect is enhanced in the present material by the stain used.) Thus in the metaphase chromosome the two daughter chromatids only are usually distinguishable, although in an occasional end view a four-parted structure is suggested (Fig. 4). Anaphasic disjunction is parallel for about one third of the inter-center distance (Fig. 5); in late anaphase, as in most coccids, each chromosome curves toward the division center (Fig. 6).

Female Meiosis

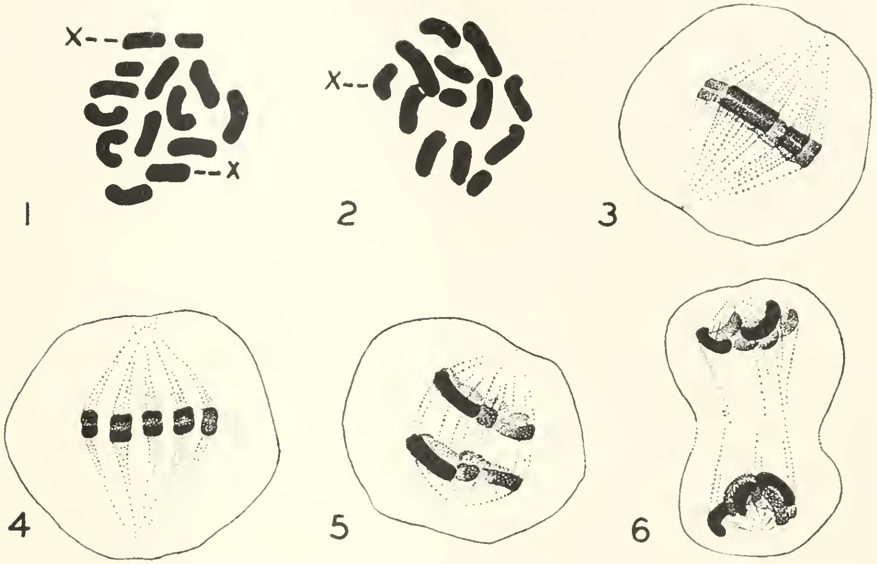
The ovary of the young female conforms in structure to the usual coccid type. There is no trace of hermaphroditism in any instar. Meiosis is completely normal throughout its course. Seven normal bivalents are formed, and invariably two polar bodies are successively given off. This has been confirmed in many eggs, from several different females. Fusion of male and female pronuclei, while both polar bodies or their derivatives are still recognizable peripherally, has been observed in several eggs. Furthermore no haploid embryos have been found among some hundred checked. Thus, although no final conclusion is justified without the confirmation of breeding experiments, all the cytological evidence indicates the absence of parthenogenesis of either diploid or haploid type.

Male Meiosis

a. Prophases; structure and orientation of bivalents.

From diakinesis on, the major features of male meiosis can be followed with adequate clarity. Earlier stages fix too poorly for a detailed analysis but appear to be entirely normal. There is no evidence of any anomalous behavior, such as vesicle formation, or a difference in rate of condensation between haploid sets of chromosomes, such as is associated with the variant degrees of asynapsis encountered in other coccids. A normal synapsis may safely be assumed. This is confirmed by the diakinetic bivalents. They are invariably six in number; no univalents other than the sex chromosome are present.

At diakinesis the autosomal bivalents and the sex chromosome are found peripherally distributed, closely underlying the nuclear membrane. The course of the constituent chromatids cannot be followed throughout the bivalents but open cross configurations (center, Fig. 7) suggest the resolution of a chiasma by rotation of the arms. Bivalent C of figure 8 would similarly be interpreted as a later stage in the same process. But the question of chiasmata aside, it is evident that in the marginally placed bivalents of figure 7 and in A and B of figure 8, the homologous chromosomes of each bivalent are assuming an end to end juxtaposition. In the interpretation of these bivalents it must be remembered that no localized kinetochore is present in these chromosomes. The median knots in bivalents such as A and B in figure 8 thus represent chromosome ends and not, as might be assumed on superficial scrutiny, kinetochores. Similarly, the large central



FIGURES 1–6. Somatic mitosis. (All drawings made with camera lucida at table level with Zeiss 2 mm., 1.3 n.a. obj. and 20 × oc.; enlarged with pantograph; magnification as reproduced 2700 ×.)

FIGURE 1. Polar aspect of metaphase, female.

FIGURE 2. Same, male.

FIGURE 3. Lateral aspect of metaphase; entire chromosome oriented, chromosomal fibers from entire length of chromosome.

FIGURE 4. Same—one focal level only drawn—showing ends of a group of chromosomes.

FIGURE 5. Early anaphase—disjunction parallel.

FIGURE 6. Late anaphase—chromosomes curve toward centers.

aperture of the bivalent separates originally sister chromatids in the vertical, and homologous chromosomes in the horizontal arms.

b. Metaphase I.

Shortening and thickening of the chromosomes proceed rapidly and with no change in the position of the homologues in relation to each other. By metaphase each bivalent is a compact, superficially four parted body; but extreme as is the condensation undergone, a polar view of an early metaphase plate (Fig. 9) still gives evidence of the end to end alignment of homologues in some of the bivalents. In the metaphase orientation, as expected under the influence of the diffuse kinetochore, the long axis of each chromosome lies at right angles to the spindle axis. The constriction visible in each bivalent from the polar view is therefore the primary split—in this case the point of contact between the ends of the two homologous chromosomes. The constriction visible from the lateral aspect (Fig 10), which becomes the plane of separation in the ensuing division, is accordingly the secondary split. The first division, patently equational for the X chromosome, is thus, disregarding crossing-over, basically equational in character for the autosomal bivalents also. In structure and orientation for the first division, therefore, the bivalents of Puto conform to the coccid and aphid type, as analysed by Ris (1942).

c. First meiotic division.

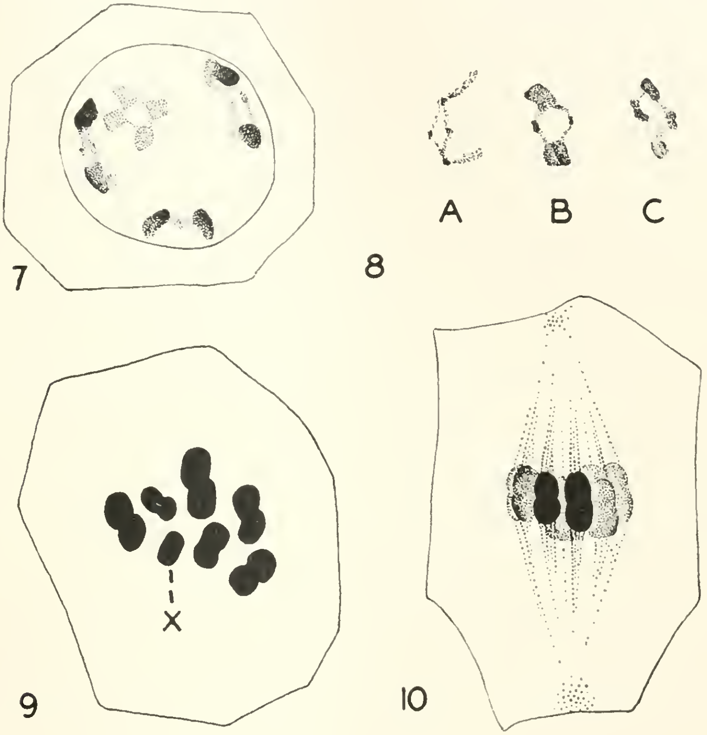
Chromosomal fibers form from the entire poleward surface of each chromatid and converge toward the division center. The four wefts of fibers thus produced in relation with each bivalent are visible in the obliquely viewed, leftmost bivalent of figure 10. No centrioles are visible and the chromosomal fibers tend to fade out distally, but the pole is nevertheless well demarcated and the spindle as a whole is of normal form. As in somatic mitosis no continuous fibers nor astral rays are present. Anaphasic disjunction is normal and regular with no trace of differential rates among the bivalents or their constituent elements. Delicate interzonal connectives form between the separating chromosomal elements, but the small size precludes an analysis of their structure.

d. Interkinesis: separation and secondary pairing of chromatids.

Already in the telophase of the first division the two chromatids derived from each metaphase bivalent begin to separate (Fig. 11). In the ensuing interkinetic interval this movement is continued until frequently the separation is complete and the full diploid number of chromatids may be counted as in figure 12. This separation is not interpretable as an extreme expression of that "repulsion" between chromatids characteristic of most organisms immediately prior to the second meiotic division. The chromatids here involved are not originally sister strands held together by a joint kinetochore region, but represent, again disregarding crossing-over, equational halves of the two homologous chromosomes of the metaphase bivalent. Their separation thus indicates simply the lapse of the terminal attraction or association which held the homologues together after the terminalization of any chiasmata which may have been present—an association ordinarily broken at first anaphase.

Little or no unravelling of the chromatids has thus far occurred. They remain throughout interkinesis as compact centers with only a slight irregularity of outline

(Figs. 12 and 13). The nuclear membrane now reforms, and it is of interest that therewith the chromatids assume once more, as previously in diakinesis and later in the spermatid nucleus, a peripheral distribution underlying the membrane. Simultaneously with this orientation the chromatids begin to reassociate in pairs (Fig. 13). Size differences show this pairing to be between homologous chromatids. Although the long axis of these compact chromatids cannot now be determined with accuracy, there is little doubt that the realignment results in a side by side lengthwise, association. This assumption is supported by the close parallellism obtaining between the realignment here and in the corresponding chromatids of Nautococcus (Hughes-Schrader, 1942) in which the long axis is persistently recognizable. Moreover, as the newly formed dyads orient for the second metaphase the plane of contact between the homologous chromatids comes to lie at right angles to the spindle axis and forms the plane of separation for the



Figures 7–10. Diakinesis and Metaphase I in male.

Figure 7. Diakinesis—(only four bivalents drawn); three bivalents show homologues assuming end to end position, one open cross.

Figure 8. Diakinetic bivalents; *A*—early assumption of end to end position of homologues; *B* and *C*—stages in opening of cross configuration.

Figure 9. First meiotic metaphase, polar view; six bivalents and univalent X; constriction in bivalents is primary split.

Figure 10. Same, lateral view; constriction in plane of separation is secondary split.

second division. Chromosomal fibers then form from the entire poleward surface of each chromatid further identifying this as the long axis. The second division is thus reductional for all non-crossover regions.

It should be emphasized that the seriation of the interkinetic stages just described can be positively established. Cell and nuclear size are in series with those of first anaphase and telophase on the one side, and second metaphase on the other. Moreover, in the first telophase alone is a heavily staining midbody developed in the interzonal connectives (Figs. 11 and 12). This midbody is retained, with decreasing sharpness of staining reaction, in the interkinetic cells and thus confirms their identification.

e. Second meiotic division.

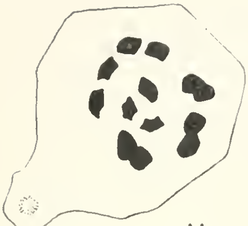
The spindle for the second division resembles that of the first, but with more sharply delimited and acuminate ends. Again chromosomal fibers alone are formed, neither continuous fibers nor astral rays being present (Figs. 15 and 16). The sex chromosome comes to lie either on the edge of the equatorial plate, or more frequently nearer to one pole, but always close to the spindle. It shows no tendency toward division, produces no chromosomal fibers, and passes usually in advance of the autosomes undivided to one pole. Second telophases show the expected two categories of spermatid nuclei—those with six autosomes and the X (Fig. 17) and those with six autosomes only (Fig. 18).

f. Quadrinucleate spermatids.

With the formation of a membrane around the spermatid nucleus the chromosomes again assume a peripheral position under it (Fig. 19). A cytoplasmic fusion of the spermatid cells in groups of four now takes place. Each resultant quadrinucleate spermatid contains, invariably, two of the six-chromosome and two of the seven-chromosome nuclei (Fig. 19). It follows that the four nuclei involved thus almost certainly represent the products of a single primary spermatocyte. The short centrally directed stalk often visible in the body of the quadrinucleate cell further suggests that the meiotic divisions may not have been quite complete cytoplasmically. If the products of the two divisions retain a connection through a common stalk a limiting factor in the fusion would be provided. Such a mechanism, it will be recalled, has been demonstrated in other coccids (F. Schrader, 1931, Hughes-Schrader, 1931). Later stages in sperm formation show the progressive development of all four components of the quadrinucleate spermatid. There is no evidence of any degeneration or loss of nuclei and apparently four normal sperm are formed from each quadrinucleate spermatid.

COMMENT

Cytologically *Puto* sp. stands out as a persistently primitive type among coccids thus far studied. This is evident in the absence of hermaproditism and of parthenogenesis and in the retention by both male and female of a normal meiosis. Its relatively generalized chromosome cycle is most nearly approached by the more primitive species of the llaveiine tribe of the family Margarodidae. Taxonomically the Margarodidae and the Ortheziidae constitute the most primitive subdivision of existing coccids; they are set off from all other families by such primitive traits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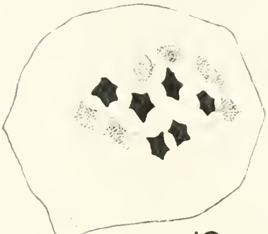
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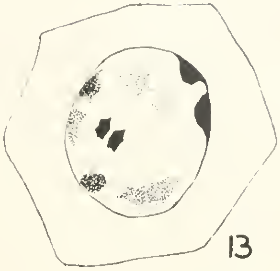
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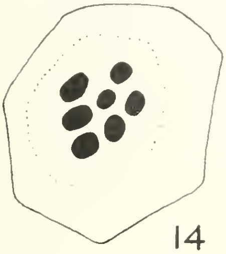
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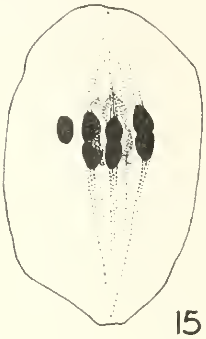
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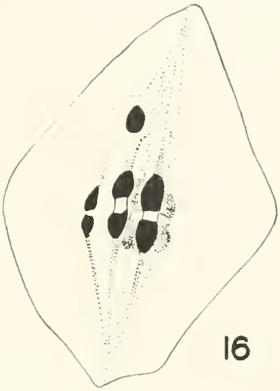
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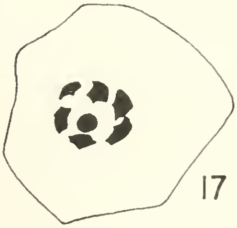
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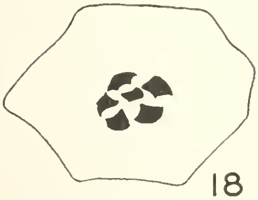
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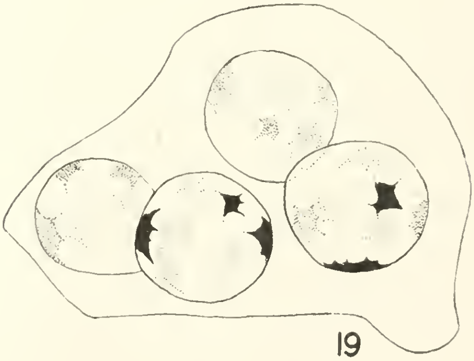
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Figures 11–19. Interkinesis and second meiotic division in male.

Figure 11. Late telophase 1 with separation of chromatids underway; spindle rest of first division at lower left.

Figure 12. Early interkinesis; complete separation of chromatids.

Figure 13. Reassociation of chromatids in pairs; spindle rest usually present at this stage not included in section.

Figure 14. Polar view of second metaphase.

Figure 15. Lateral view of same; X chromosome close to spindle at equator.

Figure 16. Early second anaphase; X chromosome near spindle, off equator.

Figure 17. Second telophase, with 6 autosomes and X chromosome.

Figure 18. Same, with 6 autosomes only.

Figure 19. Quadrinucleate spermatid, with two nuclei showing 7 and two 6 chromosomal masses.

as the retention (with a few specialized exceptions) of abdominal spiracles in all stages and well developed compound eyes in the adult males. Their closest relatives among other coccids are to be found in the Pseudococcidae—of which Puto constitutes the probably most primitive genus—linking the pseudococcid and ortheziid stems (Morrison, 1928, and personal communication).

The persistence in the family Pseudococcidae of so primitive a type of chromosome cycle as that of Puto has especial interest in view of the highly specialized male meiosis of the other pseudococcids thus far investigated. These comprise several species of Pseudococcus (Schrader, 1921, 1923a and b), and *Phenacoccus acericola* (Hughes-Schrader, 1935). These are jointly characterized by a persistent heteropycnosis of one haploid set of chromosomes in the male, by segregation without synapsis, and by the degeneration of the spermatid nuclei derived from the heteropycnotic complement. Similar conditions are encountered in *Gossyparia spuria* of the family Kermidae (Schrader, 1929) and in *Lecanium hesperidum* and *L. hemisphaericum* of the Coccidae (Thomsen, 1927, Suomalainen, 1940). While Puto throws no light on the origin of these specializations, the existence of the XX - XO sex chromosome mechanism in a primitive pseudococcid is highly significant, indicating that the male is primarily the heterogametic sex in this group. Its presence alike in Puto and the primitive llaveiines may well mean that it also represents the primitive condition for coccids as a whole. Its loss, and the substitution of alternative mechanisms—(haplo-diploidy in the Iceryini and the as yet unsolved sex determining mechanism of Pseudococcus, Phenacoccus, Gossyparia, and Lecanium in which both sexes originate from eggs fertilized by one class of sperm only)—have occurred in all other forms thus far investigated. Homogamety of the female relative to sex is indicated by the fact that in all cases the eggs of diploid-parthenogenetic females and of self-fertilized hermaphrodites (basically female in constitution) give rise exclusively to females.

The curious cytoplasmic fusion of spermatids in groups of four appears to be of very early origin in the coccid stem for it is found in every species thus far studied. Even the haploid males of the Iceryini with only one meiotic division retain the habit, producing binucleate spermatids. Multinucleate spermatids have been described in certain spiders by Wagner (1896). He reports variation within the individual and among species; the binucleate and quadrinucleate condition is frequent and higher multiples are occasionally encountered. Later authors have not dealt with the problem in detail but incidental observations (Wallace 1905, Bösenberg, 1905, and Chickering and Hard, 1935) indicate that cell bridges containing spindle remnants frequently persist between spermatids. Incomplete cytoplasmic division and multinucleate cells probably form the basis for certain of Warren's (1928, 1931) claims of amitosis in spider spermatogenesis. In the coccids no variation among species nor within the species or the individual has been observed. The fusion is always limited to the derivatives of each primary spermatocyte. The limiting factor appears to be the persistence, in the radially arranged cells of each cyst, of a centrally directed stalk from each spermatocyte—a stalk never completely severed during the meiotic cell divisions.

A significant feature of male meiosis in Puto is the complete separation and subsequent realignment of the chromatids during interkinesis. As already pointed out, this separation breaks the terminal association between homologous chromosomes which persists throughout the first division, and the realignment side by side ensures segregation at the second division. This type of meiosis, with its characteristic and essential orientation of the bivalents at first metaphase, is found in all the more primitive of the llaveiine coccids (Llaveia, Llaveiella, and Nautococcus—Hughes-Schrader, 1931, 1940, 1942). Although the meiotic figures of the female Puto are too small for critical analysis, it is significant that in the females of *Pseudococcus citri* (Schrader, 1923a) and *Lecanium hesperidum* (Thomsen, 1927), which in contrast to their highly specialized males retain an otherwise orthodox meiosis, the same separation and realignment of chromatids for the second division take place. Its occurrence in the unspecialized Puto male further confirms the conclusion that this type of meiosis is a primitive character for the coccids as a whole. Ris (1942), who first pointed out the significance of these phenomena, presents convincing evidence that the same type of meiosis obtains in aphids also, and must thus have differentiated after the Sternorhyncha had separated from the auchenorhynchous Homoptera. Incidentally it is of interest that the secondary pairing involved in this type of meiosis may well have played a role in the evolution of asynapsis among the llaveiine coccids. With the renewed operation of the pairing force just prior to the second division, asynaptic chromosomes which have divided separately and equationally during the first division, are brought together briefly at second metaphase and undergo a normal segregation. Thus in the llaveiine coccids asynapsis has been free to evolve without its usual sequelae of meiotic irregularities. Secondary pairing, while completely independent in its origin, here incidentally operates as a mechanism stabilizing asynapsis.

SUMMARY

A primitive chromosomal cycle possibly archetypal for coccids is reported for *Puto* sp. of the family Pseudococcidae. There is no hermaphroditism nor any cytological evidence for parthenogenesis. The diploid chromosome number is 14 in the female, 13 in the male. Somatic mitosis is of the type characteristic for chromosomes with diffuse kinetochore. Meiosis is regular in both sexes. In the male it can be demonstrated to adhere to the coccid-aphid type, with: (*a*) the first division equational for non-crossover regions; (*b*) separation of chromatids and their secondary pairing during interkinesis, and (*c*) segregation of non-crossover regions in the second division. An XX-female, XO-male sex determining mechanism is present. Quadrinucleate spermatids are formed. This is the only coccid thus far reported with a simple and orthodox meiosis in both sexes.

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ECOLOGICAL OBSERVATIONS ON TWO PUERTO-RICAN ECHINODERMS, MELLITA LATA AND ASTROPECTEN MARGINATUS

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I. *Mellita lata* H. L. Clark

In 1941 Dr. Henry van der Schalie collected two tests of keyhole urchins (Mellita) on the beach near Loíza Vieja, Puerto Rico. The tests were later examined by Dr. Hubert Lyman Clark,[1] who recognized them as *Mellita lata* H. L. Clark, a species recently described by him (Clark, 1940) and known previously only from two localities—Puerto Limón, Costa Rica, and La Mancha, Veracruz, Mexico. According to Clark (1933) two species of Mellita occur in Puerto Rico—*M. quinquiesperforata* (Leske) and *M. sexiesperforata* (Leske). The latter, however, is now placed in the genus Leodia Gray. The records of *M. quinquiesperforata* are all based on the collections of the "Fish Hawk" expedition which took a total of ten specimens at Ponce, Arroyo, Mayagüez, Puerto Real, and in San Juan Harbor. The collection was studied by Clark (1901) and the specimens were assigned to the species *Mellita testudinata* Klein (a synonym of *M. quinquiesperforata*) which name was, at that time, used for all five-lunuled members of the genus Mellita from the eastern coasts of the Americas.

Clark's recent revision (1940) of the genus Mellita segregates several new forms from the old group of *M. quinquiesperforata*. In the light of this critical study, the Puerto-Rican form is now to be transferred to the species *M. lata*.

This species ranks among the most common echinoderms of Puerto Rico. It occurs on sandy beaches along the entire circumference of the island. In addition to the localities listed by Clark (1901, p. 254), it has been found in the following places:

(1) Beach east of the mouth of Herrera River, east of Loíza Vieja, P. R. Several tests on the sand (coll. H. van der Schalie and the author). (MCZ No. 7972[2]).
(2) Beach about ½ mile west of Punta Embarcadero, northwest of Luquillo, P. R. Very numerous in shallow water. (MCZ No. 7997).
(3) Beach about ½ mile southwest of Punta Santiago, Playa de Humacao, east of Humacao, P. R. Dry tests on shore and living animals in shallow water. (MCZ No. 7984).
(4) Beach at Las Mareas, 4 miles southwest of Guayama, P. R. (or 1 mile

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[1] This study was begun at the suggestion of Dr. Clark, who desired to obtain additional specimens of this seemingly rare form from Puerto Rico, and asked me to gather more information on its distribution and ecology. I am grateful to Dr. Clark for his continued interest in this work.

[2] Collection of the Museum of Comparative Zoology, Harvard University.

northeast of Punta Ola Grande). Numerous specimens, close to shore (coll. Gloria Fernández). (MCZ No. 7998).

(5) Beach near Central Boca Chica, Barrio Cintrona, about 6 miles east of Ponce, P. R. Four specimens, rather fresh, dead on the beach. (MCZ No. 7986).

(6) Playa de Maní, 3 miles north-northwest of Mayagüez Harbor, P. R. Five fresh tests on the sand. (MCZ No. 7988).

(7) Beach at Punta Cadena, 6½ miles west of Añasco, P. R., in shallow water (coll. Carlos F. Blanco). (MCZ No. 7990).

(8) Columbus Park, ½ mile south of Aguadilla, P. R. Very numerous in shallow water, 1½ to 2 feet. (MCZ No. 7989).

(9) Cataño Beach in San Juan Bay, 2 miles southwest of San Juan, P. R. (MCZ No. 7999).

(10) Isla Verde, east of San Juan, P. R. Three specimens from the collection of the Department of Biology, University of Puerto Rico. (MCZ No. 7973).

In several of these localities, the animals are exceedingly numerous. This may be said, in particular, of Luquillo Beach where they were found to be most abundant close to shore, just below the zone of moving sand, at depths of from one to three feet. Up to 16 animals were counted in a square-foot area.

The people of the island coast, including fishermen, pay little attention to the animal and have no particular name for it. In two places, Humacao and Aguadilla, I heard them referring to Mellita as "estrella" which means star and is also the name used generally for sea-stars. Apparently the use of the name is due to the radial pattern of the oral surface.

A general description of the morphology of *Mellita lata* was given by Clark (1940, pp. 437–438, and pl. 60, fig. 1; pl. 61, fig. 1; pl. 62, figs. 1, 2). The species is characterized mainly by its elliptical shape, the width exceeding the length considerably; by the anterior situation of the apex; by the dimensions and the shape of the lunules; and by the large heads of the capitate aboral primary spines.

Color of the living animal.—The aboral surface is dark grayish olive-green. The oral side (Fig. 2) has a remarkable color pattern. In animals from Luquillo, the ambulacral areas are usually dark wine-red, occasionally dark purple, or more rarely a lighter shade of red. Lighter (pinkish), narrow, somewhat branched bands radiate from the peristomial margin towards the inner ends of the paired lunules (I, II, IV, and V) and similarly in the anterior midline (III) towards the anterior margin. More irregular light patches and stripes, extending in a transverse direction, occur on both sides of the unpaired lunule. In specimens taken at Aguadilla, however, the ambulacral areas were brown, the shade ranging from deep yellow-brown to red-brown, and the lighter bands and patches were in a light brown hue. The interambulacra are covered with silvery, translucent spines and appear whitish or light pink.

The color of the dark areas of the oral surface is due mainly to the coloration of the numerous tube feet and that of the periproct with the anal tube. In other places, the epidermis has a light pink or yellowish color.

After the spines and the epidermis of a fresh specimen are removed, the following

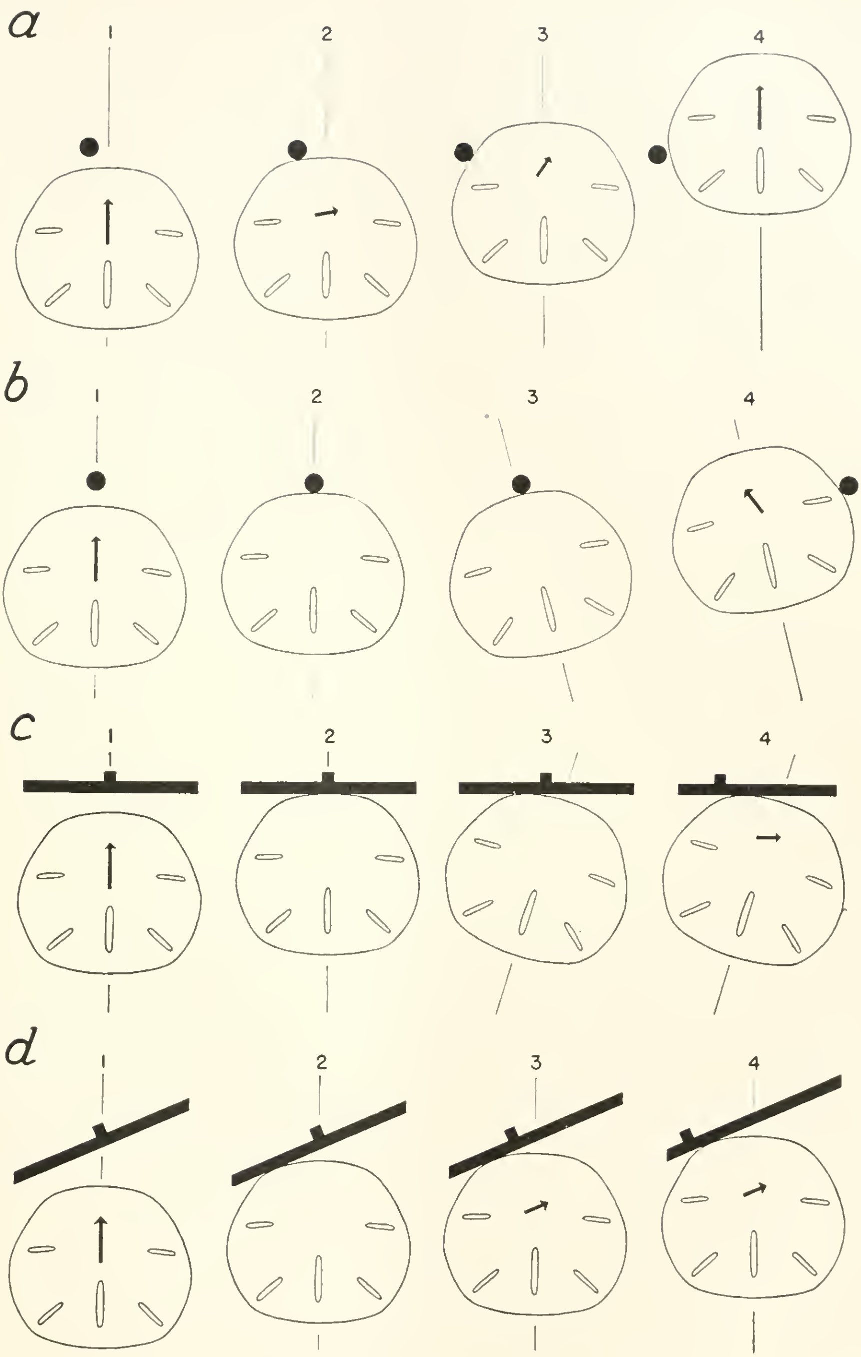


FIGURE 1. Behavior of *Mellita lata* meeting obstacles while digging into the sand: (*a*), successive positions at running against stick placed vertically in the path of movement, to one side of the midline; (*b*), stick placed vertically in the center of path; (*c*), vertical board placed transversally to the direction of movement; (*d*), vertical board placed obliquely in the path of movement.

color pattern is seen on the exposed test: Aboral side—blue-green; oral side—ambulacral areas brown and the interambulacra white, pink, or light yellow.

The dark coloring matter of the oral side is extracted by the common preservatives (alcohol, diluted formalin) in a very short time.

The anus is situated as the end of a short tube (about three millimeters long in fully grown animals) and is surrounded by small, irregular papillae. The tube feet are closely packed in the ambulacral areas, except in the radial bands of lighter color. Moreover they are found along the margins of all lunules, including the posterior or interambulacral lunule, and also among the marginal spines of the entire circumference.

The oral side has three kinds of spines which may be roughly grouped as follows: Long, slender spines of the interambulacra (these are the ones responsible, together with the marginal spines, for the locomotion of the animal); medium-sized spines scattered over the ambulacral areas; and short spines of the ambulacral surfaces, particularly flanking the ambulacral furrows. No pedicellariae were seen.

Ecology.—The animals live buried in the uppermost layer of the sand in such a manner that usually only the posterior lunule, with a small part of the posterior surface and of the posterior margin, is visible. Occasionally, also lunules I and V may be exposed. Only exceptionally is the contour of the entire animal discernible. They move continuously through the sand, their speed varying from 11 to 26 millimeters per minute.

Experiments on locomotion.—Several individuals were placed on a layer of sand in a flat pan filled with sea water. At first the animals remained quiet and no movement of the marginal spines was seen from the aboral side. After a short time—a few seconds up to perhaps one minute—they began to move forward, first in small jerks and later in continuous movement. They dug obliquely into the sand and disappeared from the surface within a short time, in from one to about four minutes (see Table I and Figs. 4*a* to 4*c*).

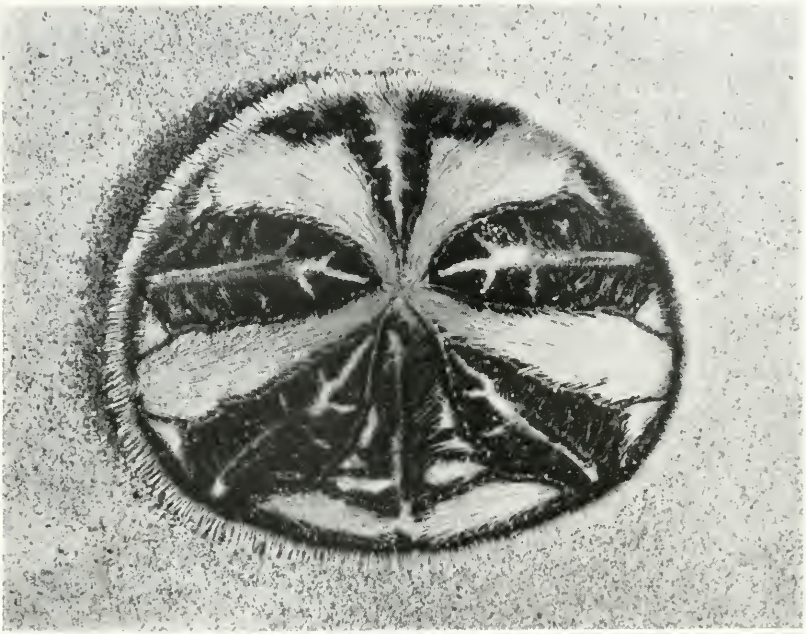
TABLE I

Time required by Mellita lata *to dig in completely. Temperature, 28° C.*

Specimen	Length in millimeters	Time required	
		First trial	Second trial
A	25	1 min. 20 sec.	1 min. 30 sec.
B	26	1 min. 25 sec.	
C	36	2 min. 30 sec.	1 min. 45 sec.
D	53	2 min. 05 sec.	
E	60	2 min. 40 sec.	
F	70	3 min. 20 sec.	3 min. 20 sec.
G	71	3 min. 05 sec.	

As the animals move in a forward direction, they need a "runway" of a certain length to dig in successfully. Two specimens placed in a jar with a diameter of about 90 millimeters, were moving around continuously, for hours, but were always on the surface of the sand.

During the digging process, the animals do not react to such mechanical



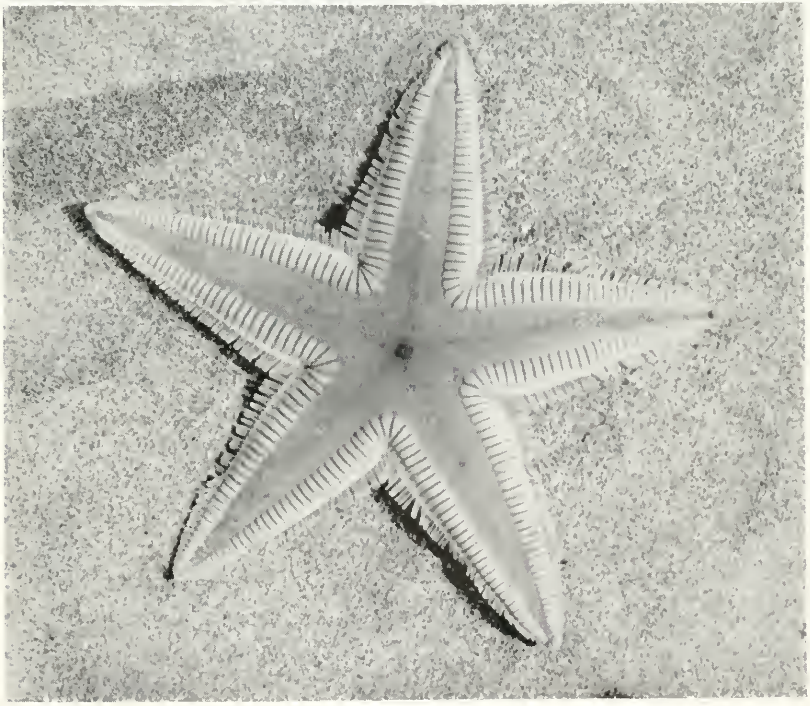


Figure 2. *Mellita lata* H. L. Clark, oral side, natural size.
Figure 3. *Astropecten marginatus* Gray, aboral side, ⅘ natural size.

stimuli as tapping the exposed surface with a wooden stick, but continue their locomotion without interruption and at an even rate. If the tapping is so strong as to dislodge the animal from its hold in the sand, the movement is stopped for a short time, to be soon again resumed in the usual way.

The following four experiments have been conducted repeatedly to determine the behavior of a digging animal meeting obstacles placed in its way:

(*a*) If a stick is placed vertically before the animal, to one side of the midline (Fig. 1*a*), locomotion is almost stopped when the animal reaches the obstacle, but the rhythmical movements of the spines continue. Slowly the animal works its way around the stick keeping its original orientation all the time (*i.e.*, the antero-posterior axis remains parallel to the original direction of motion as the animal shifts to the right or left, depending on the location of the obstacle with respect to the midline).

(*b*) When a stick is placed vertically at the center of the frontal margin (Fig. 1*b*), the animal at first stops its locomotion, but continues to move its spines. Within a short time, the pushing of the spines of one side—the right or the left—prevails and the animal turns slowly towards the weaker side. It then continues to move with an orientation of the axis which is at a slight angle to the original direction.

(*c*) If a vertical board is placed transversely in the path of movement (Fig. 1*c*), the animal continues the movement of the spines upon reaching the plane. Slowly it turns to the right or left, for approximately 16 degrees. Then it continues moving laterally along the plane, at a very slow rate, but retains the axis constantly in the new orientation.

(*d*) When a vertical board is placed obliquely (at an angle of less than 74 degrees) in the path of the movement (Fig. 1*d*), the animal continues to move, at very slow speed, towards the open side, without rotating its antero-posterior axis.

If two animals collide, head-on, while digging into the sand, the stronger individual may push the weaker one back for a short distance. Finally, one of the animals usually crawls over the top of the other. It is obvious that, on account of the great density of the Mellita population on some beaches, such meetings must be of very frequent occurrence.

If an animal is placed on the sand, oral side up, in quiet water (e.g., in a shallow dish), it is capable of very slow forward movement, but it neither can turn into its normal position nor dig into the sand. In moving water, however (in the wave zone of the beach), it is soon turned over by the current and then remains in its right position. This recovery is obviously due to the general shape of the body (concave oral surface, convex aboral side).

One specimen of *Mellita lata* was placed in a glass dish containing sea water and a very thin layer of sand, so that it could be observed from both the oral and aboral sides. It could be seen that the locomotion is accomplished by rhythmical movements of the long spines of the body—the marginal spines, spines of the interambulacra 1, 2, 3, and 4, and the spines of the fields on both sides of the posterior lunule (5). The movement of the spines in each area is metachronal, progressing like a wave in a wheat field. The rate of beating is approximately the same in all areas, about 35 strokes per minute in the animal examined (at 26.5° C.).

Little is known so far about the ecology and the locomotor activities of allied

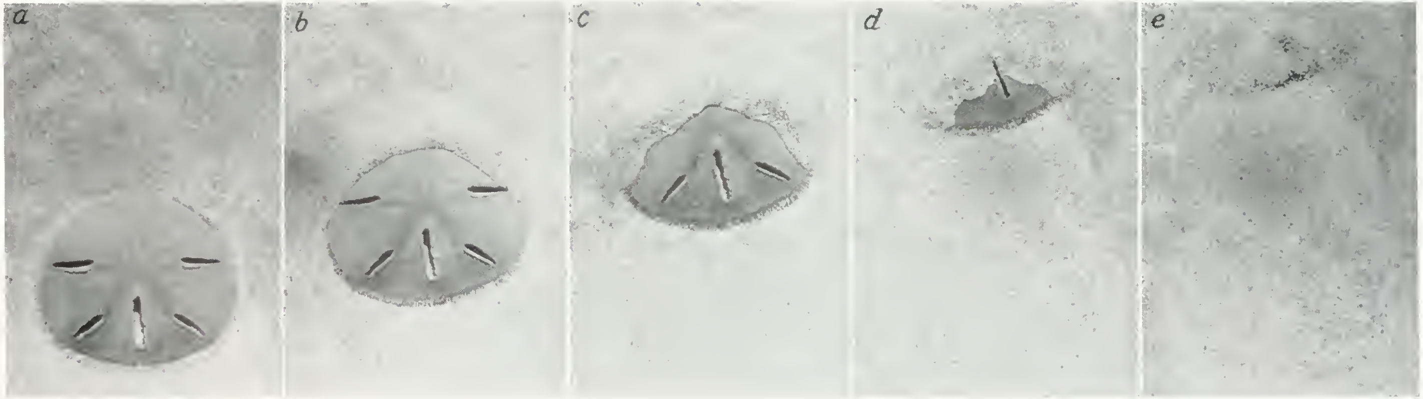


FIGURE 4. Photographs of *Mellita lata* digging into the sand, taken at various times after the beginning of the movement: (*a*), after 10 seconds; (*b*), after 30 sec.; (*c*), after 55 sec.; (*d*), after 105 sec.; (*e*), after 140 sec.

species of the family Scutellidae or sand-dollars. Observations by Pearse *et al.* (1942, p. 150) on *Mellita quinquiesperforata* (Leske) at Beaufort, North Carolina, indicated a behavior rather different from that exhibited by our species: "The sea urchins, Moira and Mellita, also move almost directly downward by rapidly waving their spines and tube feet so as to move sand from underneath their tests toward the margins; then move it from the margins so as to cover the upper surface; neither sea urchin makes progress anteriorly as it descends."This mode of movement appears, however, to be exceptional among the sand-dollars. Generally they burrow in a forward direction as *M. lata* does. This method of burrowing has been observed in *Leodia sexiesperforata* (Leske) by Crozier (1920); in *Echinarachnius parma* (Lamarck) by Parker (1927) and Parker and van Alstyne (1932); and in

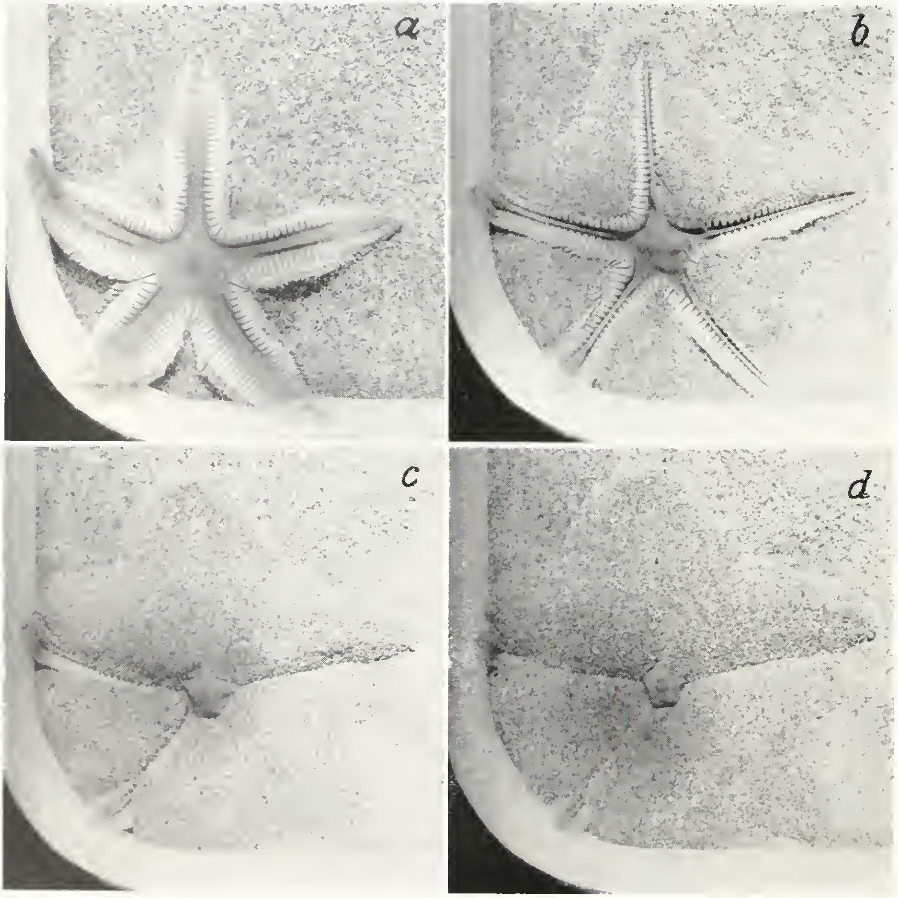


FIGURE 5. Photographs of *Astropecten marginatus* digging into the sand, taken at various times after the beginning of the movement: (*a*), at start; (*b*), after 25 seconds; (*c*), after 45 sec.; (*d*), after 65 sec.

E. mirabilis (A. Aggassiz) and *Astriclypeus manni* Verrill by Ikeda (1939, p. 77). In the speed of burrowing, *M. lata* exceeds all its relatives that have so far been observed. *Leodia sexiperforata* is reported to disappear in the sand in "less than 15 minutes" (but may require two or three times that long), and *Echinarachnius parma* in 10 to 20 minutes.

Rotating movements, either a turning movement with the mouth as center, or a swinging of the anterior end to the right or left during forward locomotion, have been reported in all species so far studied. *M. lata,* however, seems to lack this type of movement, or at least does not exhibit it to a noticeable degree. The body axis is turned only when the animal encounters an obstacle to its forward movement. Horizontal locomotion in a backward direction, such as Ikeda reported for *Echinarachnius mirabilis* and *Astriclypeus manni,* has not been observed in *M. lata.* Our form also lacks the ability to right itself after having been placed on the sand oral side up, while related species generally possess this ability.

One is tempted to correlate the shape of the test of *Mellita lata* with the normal locomotion of the animal, in the uppermost layer of the sand. In a median section through the animal, the greatest height is seen nearer to the anterior margin. The median section thus has a "streamlined" contour. This is obviously the contour that offers the least resistance to the forward movement. With regard to this streamlined shape, the species *lata,* as well as *M. longifissa* Michelin and *M. latiambulacra* H. L. Clark in which the general form is similar, appear to be more highly specialized and better adapted to their peculiar way of living, than are other species of the genus Mellita in which the apex has a more central position.

II. *Astropecten marginatus* Gray[3]

Clark (1933, pp. 16-19) listed two species of *Astropecten* from Puerto Rico, *A. duplicatus* Gray and another species that he had originally (1901) called *A. antillensis* Lütken, but that he later was inclined to consider identical with *A. articulatus* (Say). While observing *Mellita lata,* I found, in the same habitat, *A. marginatus* Gray, a species hitherto unrecorded in the Greater Antilles. It was identified by H. L. Clark.

A. marginatus (Fig. 3) was repeatedly collected at Luquillo Beach, about one-half mile west of Punta Embarcadero, northwest of Luquillo, Puerto Rico. It lives there buried in the sand and shares its habitat with the much more abundant keyhole urchin. It does not come as close to shore as Mellita does, but is more numerous in the deepest water accessible by wading (four to five feet). In the collection of this sea-star I was very ably assisted by Mr. Víctor A. Marcial. The animals were obtained by probing the sand with the hands or feet while wading. Only a few were seen on the surface of the sand. Several specimens were deposited in the Museum of Comparative Zoölogy, Cambridge, Massachusetts (Nos. 4109, 4110, 4113 and 4114).

The known geographic range of *A. marginatus* comprises the coasts of Venezuela, Trinidad, Guiana, and Brazil as far south as 27° 30′. It appears, therefore, probable that the species reached Puerto Rico via the Lesser Antilles, an assumption that Dr. Clark would extend also to *Mellita lata.*[4]

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[3] Gray, 1840, *Ann. Mag. Nat. Hist.*, **6**: 181.

[4] Personal communication.

Color in life.—In a great majority of about 30 specimens collected, the aboral side was a beautiful blue-green, of various shades, darkening towards the tips of the arms. This color extended both over the superomarginals (with their marginal spines) and the paxillar area. The center of the disk had a small, round brownish spot. Only two individuals exhibited an aberrant coloration, a yellow-brown aboral surface. The oral side is creamy white, with the ambulacral feet showing a very faint tint of brown.

The blue-green coloring matter of the aboral side is remarkably unstable. When animals were placed in alcohol, they rapidly turned brown-yellow. This change was clearly visible within one minute. Later the brownish color dissolved and stained the alcohol, while the specimens became white. In diluted formalin the color is altered more slowly, but is lost within a few days. It is possible that the green and brown coloring materials represent only modifications of the same pigment. If so, the two brown specimens mentioned may not be as strikingly aberrant as they would otherwise appear to be.

Freshly caught specimens, placed in a dish filled with sea water and sand, move at first rather fast over the surface of the sand, preferably along the walls of the dish. In this movement, the body is lifted up and supported by the tube feet in the way repeatedly observed in other species of Astropecten (Romanes and Ewart, 1882, p. 839, etc.).[5] Later, they come to rest, usually in a corner. After staying there for a time, they begin to dig into the sand, perpendicularly and simultaneously with all five arms. Figures 5*a* to 5*d* show successive stages of this digging activity. The process is surprisingly fast and the animal photographed would have been completely covered within one minute, had it not reached the bottom of the dish and come to rest when the ends of the arms and the central part of the disk were still exposed.

The digging is done by movements of the tube feet. At first, the margins of the arms are bent upwards rather steeply, so that the marginal spines assume a vertical position. The paxillar area of each arm is folded into a deep furrow and the arm becomes very narrow. Then sand is seen coming up from the deeper layers along the margins of the arms.

SUMMARY

The common Puerto-Rican keyhole urchin, *Mellita lata* H. L. Clark, and the sea-star, *Astropecten marginatus* Gray, live in the uppermost layers of the sand of shallow beaches. The color in life, and various locomotor activities of the two species are described.

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[5] The speed of this "running," though not actually measured, exceeds considerably the velocity of "between one and two feet per minute" that Romanes and Ewart record for *A. aurantiacus* (L.).

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THE FOOD-VACUOLE IN THE PERITRICHA, WITH SPECIAL REFERENCE TO THE HYDROGEN-ION CONCENTRATION OF ITS CONTENT AND OF THE CYTOPLASM

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INTRODUCTION

The food-vacuole in the Peritricha was probably first seen in *Vorticella* sp. by Ehrenberg in 1830. Since then it has been observed in many different species by various investigators but it has not been intensively studied in any. There are consequently a number of unsolved problems concerning it. Some of these problems are considered in the following pages.

MATERIAL

Observations were made on the following species: *Epistylis plicatilis* (Ehrenberg), *Campanella umbellaria* (Linnaeus), *Campanella tincta* (Stokes), *Vorticella microstoma* (Ehrenberg), *Vorticella similis* (Stokes), *Vorticella convallaria* (Linnaeus), *Vorticella campanula* (Ehrenberg), *Carchesium epistylis* (Claparede and Lachmann), *Zoothamnium arbuscula* (Ehrenberg) and *Ophrydium ectatum* (Mast, 1944).

It was found that the internal structure can be more clearly seen in *Vorticella similis* and *Campanella umbellaria* and *tincta,* than in any of the other species. These three species were consequently much more thoroughly studied than the others. The specimens of *Campanella tincta* used were collected by Dr. A. Dawson in a pond containing much *Elodea* in the vicinity of New York City and shipped to Woods Hole where they lived well in the laboratory for more than a week. Nearly all were attached to the stems and leaves of *Elodea* and most of them were single. *Campanella umbellaria* was found in abundance in a shallow ditch in a peat marsh adjoining a small lake known as Sol's pond about one mile northeast of Falmouth, Mass. The water in this ditch was covered with duckweeds (*Lemna*) and was distinctly acid (pH 6.2) but clear. Ehrenberg (1838) and Greeff (1870–71) called this organism *Epistylis flavicans*.

The two species of *Campanella* studied were practically the same in form, gross structure, size, formation of colonies and behavior, but the former had six double rows of cilia on the peristome, was grayish in color, owing to numerous conspicuous granules, and was found chiefly on *Elodea*, while the latter had only four double rows of cilia, was distinctly yellowish in color, had no conspicuous granules, and was found chiefly on *Lemna*.

Vorticella similis was found in abundance attached to duck-weeds in a pond which contained all sorts of refuse including much ashes. The water in this pond was continuously distinctly alkaline, usually pH 8.2. Most of the specimens used were, however, obtained from laboratory cultures. They thrive indefinitely in boiled tap-water containing crushed hemp seeds (two seeds in 50 cc. in a finger bowl) if the solution is renewed about once a week. In the pond they were always found very near the surface and in the laboratory they grew well only in shallow water. They apparently require an abundance of oxygen.

Structure of the Feeding Apparatus

Introduction

All observers agree that the feeding apparatus in the Peritricha contains a ciliated tube which is connected with the peristome, that this tube consists of an outer part in which the cilia produce an ingoing and an outgoing current and an inner part in which they produce only an ingoing current, and that the fecal substance and the content of the contractile vacuole are discharged into the outer part. There is, however, much variation in the names applied to these two parts and great diversity of opinion concerning the structure of the cytoplasm beyond the distal end of the inner part, as set forth in the following paragraphs.

The outer part is called "buccal cavity" by some, "vestibulum" by others and "vestibule" by still others. The outer opening of this part is called "mouth" by some, and the inner opening is called "mouth" by others. The inner part is called "pharynx" by some, "cytopharynx" by others and "oesophagus" or gullet by still others. Some hold that it opens directly into the cytoplasm, others that it does not. We shall call the outer part "vestibulum," the inner part "pharynx," and the outer opening of the vestibulum "mouth."

Ehrenberg (1838) concludes, on the basis of the direction of the movement of the food-vacuoles through the cytoplasm, that the pharynx opens into a tube or gut which extends through the cytoplasm to the anus in the wall of the vestibulum. Koehring (1930, p. 55) supports this conclusion. She could not see a differentiated tube in the cytoplasm but she says that the "orderly course" of food-vacuoles in *Vorticella* sp., and other evidence, indicates that there is a "digestive system in ciliates, comparable to the digestive system of many metazoan organisms." Greeff (1870) could find no evidence of a digestive system in the peritricha but he main-

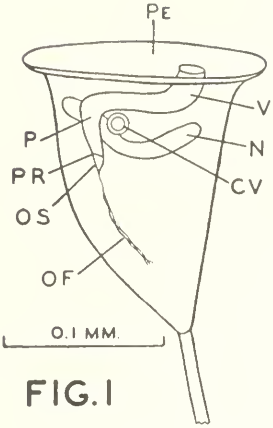
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FIG. 1

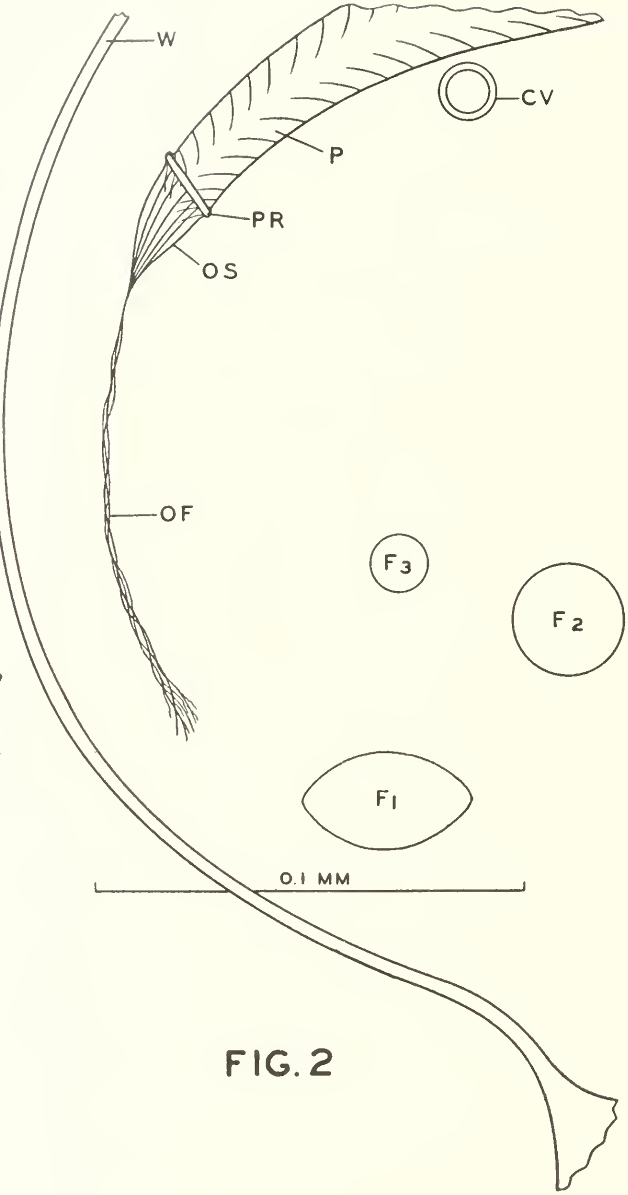
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FIG. 2

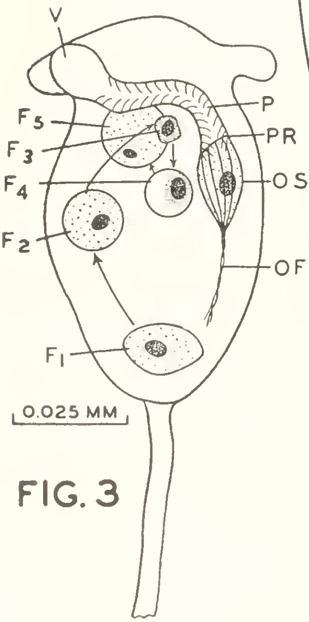
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FIG. 3

FIGURE 1. Camera outline of *Campanella umbellaria*. *Pe*, peristome containing six double rows of cilia, not shown; *V*, Vestibulum; *P*, pharynx; *PR*, pharyngeal ring; *OS*, oesophageal sac; *OF*, oesophageal fibers; *N*, nucleus; *CV*, contractile vacuole.

FIGURE 2. Camera sketch of a portion of the feeding apparatus in *Campanella umbellaria* greatly compressed. *P*, pharynx; *PR*, pharyngeal ring; *OS*, oesophageal sac; *OF*, oesophageal

tains that in at least some of them (*Campanella umbellaria*) the pharynx opens into a funnel-shaped structure ("der Trichter") which in turn opens into a tube ("Oesophagus") but that this tube opens directly into the cytoplasm near the posterior end of the body, not into the vestibulum near the anterior end. Schröder (1906) says he observed such a tube in *Epistylis plicatilis* and *Vorticella monilata* as well as in *Campanella umbellaria* and Kahl (1935) concludes that it is present in all the Peritricha. Greenwood (1894) and Kitching (1938) were however unable to find any indication of it in any of some ten species studied.

Material and methods

Observations were made on the structure of the feeding apparatus in all the species listed above but certain parts of it could be more clearly seen in the two species of *Campanella* than in any of the others. No difference was found in the feeding apparatus in these two species. They were consequently used indiscriminately. Both contain so much opaque substance that their internal structure cannot be made out under normal conditions. It was found, however, that, owing to their tough elastic surface membrane, they can be greatly compressed without injury and that this greatly facilitates observations on their structure.

The observations were made as follows: A small unattached colony in tap-water containing a little powdered carmine, was mounted under a cover-glass supported by two small parallel ridges of vaseline. Water was then very slowly removed with a strip of filter paper until the campanellae were compressed as much as desired. During this process they were closely observed under low and high magnification. In some preparations the organisms were fixed by drawing Schaudinn fluid and alcohol under the cover-glass. However, this did not facilitate observations on the structure. Compensating oculars (10, 15 and 20x), apochromatic objectives (10, 20 and 40x dry and 60x oil immersion, n. ap. 1.4), an achromatic condenser and a concentrated filament lamp, with ground glass ray-filter and an iris diaphragm, were used in all the observations.

Results

The results obtained are presented in Figures 1 and 2 and the following paragraphs: These figures show that the wall of the pharynx in *Campanella* is considerably thicker at the distal end than elsewhere and that attached to this end there are several fibers which converge as they proceed and soon form a bundle which extends through the cytoplasm nearly to the posterior end of the body. The thickened end of the wall of the pharynx forms a definite ring which is highly refractive and distinctly yellowish in color. We have designated it the pharyngeal ring and the fibers attached to it, the oesophageal fibers (Fig. 2). The oesophageal fibers can be seen near the ring only under occasional circumstances and then not

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fibers; *CV*, contractile vacuole; F_1-F_3, food-vacuoles, showing change in shape and size; *W*, membrane at the surface of the body.

FIGURE 3. Camera outline of an optical section of *Vorticella similis*. *V*, vestibulum; *P*, pharynx; *PR*, pharyngeal ring; *OS*, oesophageal sac; *OF*, oesophageal fibers; F_1-F_5, food vacuoles, showing change in form and size; small dots, bacteria and granules; large dots, yeast-cells (The body contains numerous food-vacuoles and granules not represented).

very distinctly. They can however be seen definitely in the bundle but they cannot be clearly differentiated because they are superimposed and close together. Seven were definitely seen in one bundle and three to five in others. There probably are a few more than seven and they probably are equally spaced in their attachment to the pharyngeal ring. In some specimens the bundle was spread out considerably at the end forming a brush. In specimens which have been compressed and killed under a cover-glass the oesophageal fibers remain intact for several days if the preparation is sealed with vaseline and kept in a damp chamber and they do not decrease appreciably in distinctness for at least two days.

No activity was seen in the oesophageal fibers except in one specimen. This specimen was greatly compressed. The cytoplasm in it had gathered around an irregular cavity at the end of the pharynx. The cilia in the pharynx were still active and were forcing fluid into this cavity, which was abnormally large so that the oesopageal fibers were much distorted in their arrangement and in their connection with the pharyngeal ring. Three of these fibers, only slightly separated from each other, extended through this cavity near one side and then joined the rest in the bundle. Waves were definitely seen to pass synchronously along these three fibers, from their attachment to the ring, on into the bundle. This activity continued, however, only a few moments after which the entire organism appeared to be dead.

Numerous attempts were made to reproduce the conditions under which this was seen but without success. In one specimen, however, in which an irregular cavity had formed at the end of the pharynx, six inactive oesophageal fibers were seen to extend from the pharyngeal ring through the cavity. The physiological state necessary for activity in these fibers probably continues such a short time after the campanellae are compressed that it is rarely encountered.

Oesophageal fibers were seen in all the other species studied and a pharyngeal ring in several. The fibers were fairly distinct in *Vorticella similis* (Fig. 3), *Epistylis plicatilis* and *Ophrydium ectatum* (Mast, 1944), but they could not be counted with certainty in any of them, although seven were distinctly seen in one ophrydium and five in one vorticella. There doubtless are more, probably about ten.

Numerous specimens of *Vorticella similis* were fixed (some in hot Schaudinn and others in hot Bouin fluid) stained with Heidenhain haematoxylin, and sectioned (3, 5, 7 and 10μ). Those fixed in Bouin fluid were much better than those fixed in Schaudinn, but the oesophageal fibers could not be as distinctly seen in either as in living specimens.

There was no indication of an oesophageal tube in any of the species studied. If there actually is such a tube the fibers observed must be in its wall. There is, however, considerable evidence (presented later) which opposes this supposition. There is, then, in the results obtained no support for the views of Ehrenberg and Koehring or Greeff and Schröder presented above.

Fibers extending from the pharynx have been seen by Schuberg (1890) in *Stentor,* Sharp (1914) in *Diplodinium,* Andrews (1923) in *Folliculina* and Bozler (1924) and Lund (1941) in *Paramecium.* Schuberg and Andrews maintain that the fibers are in the wall of an oesophageal tube. Sharp, Bozler and Lund maintain that they extend directly through the cytoplasm. The views concerning their function vary greatly.

Formation and Movement of the Food-vacuoles

It is well known that in the peritricha the food-particles aggregate at the distal end of the pharynx, but opinions differ as to how the food-vacuoles are formed and transported through the cytoplasm.

Numerous observations were made on the process of feeding in many specimens of *Campanella umbellaria* and *tincta* and *Vorticella similis* under various conditions, and on a few specimens of each of the other species listed above. The results obtained in the observations on *Campanella* led to the following conclusions:

When the organisms are not feeding there is at the distal end of the pharynx a cone-shaped space filled with culture fluid and particles suspended in it. At the surface of this space there is a membrane in the form of a cone-shaped sac which we shall call the oesophageal sac (Fig. 2). This membrane is doubtless produced by the interaction between the fluid in the space and the adjoining cytoplasm. Pharyngeal cilia project into the sac and the oesophageal fibers pass from the pharyngeal ring over its surface to its apex where they unite to form a bundle which passes on into the cytoplasm. When feeding begins the pharyngeal cilia force more culture fluid and particles into the oesophageal sac. This stretches the membrane around it, but continuous interaction between the fluid in it and the adjoining cytoplasm prevents this membrane from becoming too thin. As the sac enlarges it becomes spindle-shaped, owing to unequal pressure of the oesophageal fibers and possibly the adjoining cytoplasm on different regions of its surface. Under normal conditions enlargement continues until the sac is nearly twice as wide as the pharynx, then a constriction begins to form near the pharyngeal ring. This constriction increases until a spindle-shaped portion of the sac is pinched off, leaving a cone-shaped portion attached to the pharynx, the same in shape and size as that which obtained before feeding began (Fig. 4 *A–E*). The spindle-shaped portion is a new food-vacuole. There is no perceptible change in size of the pharynx or the pharyngeal ring during this process. These structures are consequently not directly involved in the formation of the food-vacuole.

The newly formed food-vacuole moves rapidly through the cytoplasm to the distal end of the oesophageal fibers. Here it remains a few moments, usually turning sharply, then it proceeds slowly with the cytoplasm on an indefinite course, ending in the lower part of the vestibulum where its indigestible content is discharged. Its slow movement is obviously due to the movement of the cytoplasm in which it is suspended, i.e. to cytoplasmic streaming, but during its rapid movement definite currents are produced in the adjoining cytoplasm, showing very clearly that this movement is not due to cytoplasmic streaming.

The constriction in the oesophageal sac is probably due to simultaneous inward pressure, in the same region, of the oesophageal fibers on its surface; and the food-vacuole is probably transported from the pharynx to the posterior end of the body within the bundle of oesophageal fibers by waves passing synchronously along these fibers and from the posterior end of the body to the vestibulum by streaming movement in the cytoplasm (cyclosis).

The formation and transportation of the food-vacuoles in *Vorticella similis* and all the other species studied is in full harmony with this description. In all, the food-vacuole is formed by pinching off a portion of a cone-shaped sac attached to the pharynx and in all the food-vacuole is spindle-shaped and passes rapidly

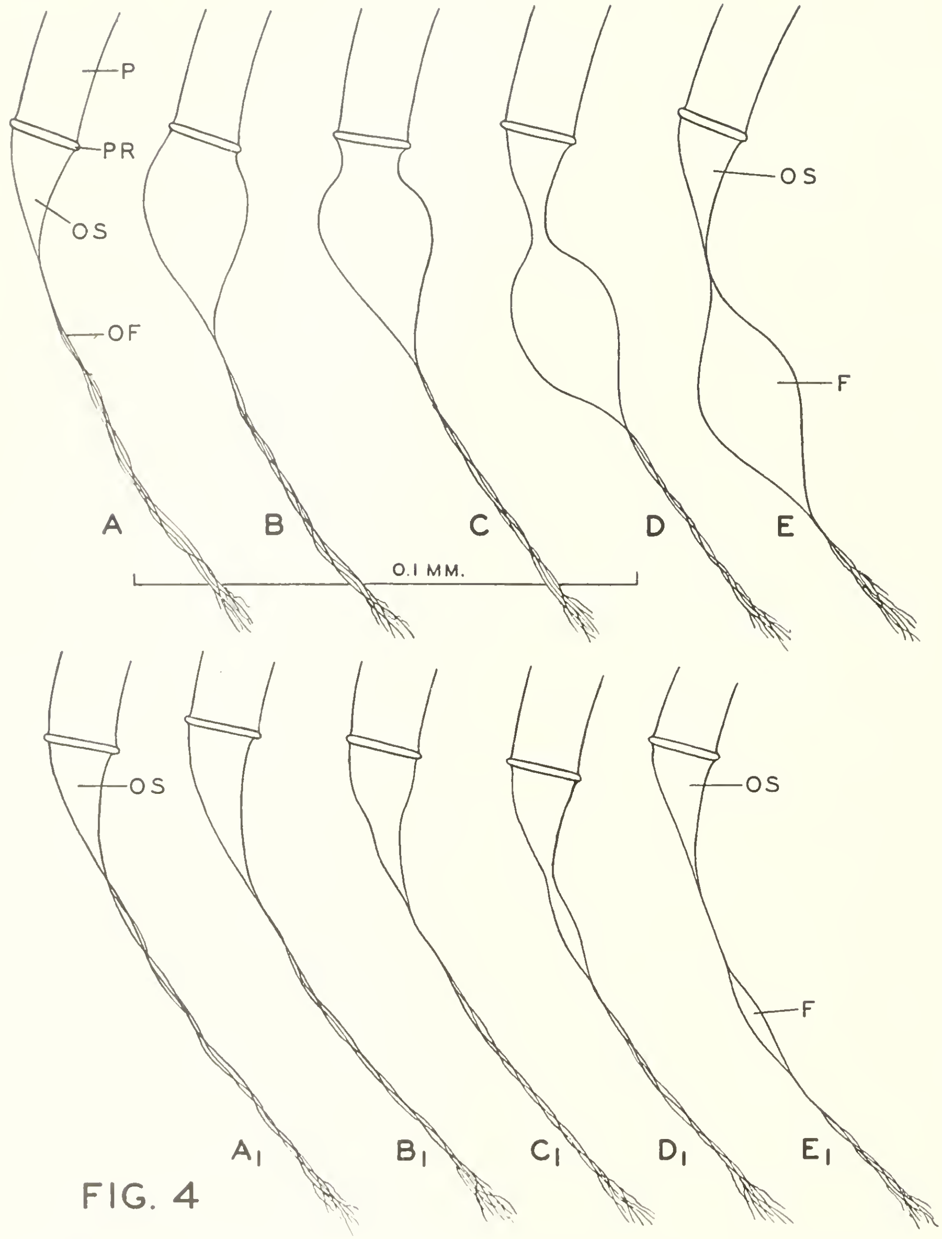

FIG. 4

Figure 4. Outlines showing a portion of the feeding apparatus and the formation and movement of food-vacuoles in *Campanella*. *A* and A_1, feeding apparatus in a specimen not feeding or immediately after a food-vacuole has been formed; *B-E*, successive stages in the formation of a food-vacuole under normal conditions; $B_1 - E_1$, same in a compressed individual with peristome closed and its cilia inactive. *P*, pharynx; *PR*, pharyngeal ring; *OS*, oesophageal sac; *OF*, oesophagel fibers; *F*, food-vacuole.

Note that under normal conditions the cone-shaped oesophageal sac enlarges greatly and through the cytoplasm to the posterior end of the body and then slowly with the cytoplasm through the body. No evidence of an oesophageal tube was observed in any of these species.

These conclusions and others are strongly supported by the results obtained in detailed observations on variations in the formation and the movement of food-vacuoles in several specimens. These observations are considered in the following paragraphs:

1. In a specimen of *Vorticella similis* mounted in tap-water but not compressed, it was observed that the food-vacuoles had, immediately after they were formed, a long projection at one end. One of these vacuoles was continuously studied under the oil-immersion objective during the entire process of formation and for some time after, and the following observed:

The constriction in the oesophageal sac did not completely separate the food-vacuole from it. When the vacuole moved away this connection was drawn out until it had formed a strand fully as long as the vacuole; then it broke at the apex of the sac. The vacuole with this strand attached now moved rapidly to the posterior end of the body, then turned sharply; after which the strand folded over, came in contact with the surface of the vacuole, and fused with it; then the vacuole moved on slowly and very slowly rounded up. The membrane on the surface of this vacuole appeared to be very thick and viscous.

In other specimens of this species under the same conditions, but with powdered carmine added to the tap-water, some of the food-vacuoles remained spindle-shaped for at least one hour after they had reached the posterior end of the body and in some specimens of *Ophrydium ectatum* more than two hours, whereas they ordinarily round up in a few moments. Obviously either the membrane at the surface of these vacuoles was thicker and more viscous than ordinarily or their entire content was more viscous, probably the latter.

The results presented above show that constriction in the oesophageal sac is not the only factor involved in the formation of the food-vacuoles, that is, that in connection with this constriction there must be a mechanism which forces the vacuole toward the posterior end of the body so as to stretch out and break its connection with the oesophageal sac. They also show that the membrane at the surface of the food-vacuole is formed while it is still a part of the oesophageal sac, not after it has reached the posterior end of the body as some maintain. They show, moreover, that the membrane at the surface of the vacuole varies greatly in thickness and in viscosity and that the entire content of the vacuole probably also varies greatly in viscosity.

2. A specimen of *Campanella umbellaria* was greatly compressed and then continuously observed under the oil-immersion objective. The peristome was inverted and the cilia on it were inactive but those in the vestibulum and the pharynx were active and food-vacuoles were formed at intervals of about 45 seconds; but after nine had been given off all ciliary action ceased. All these vacuoles were spindle-shaped, but much smaller and relatively much longer than those formed under

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becomes spindle-shaped, that a portion of this sac is constricted off to form the food-vacuole, and that the constriction begins at the base of the sac near the pharyngeal ring; but that under abnormal conditions the sac enlarges but little, that only a small portion is constricted off and that the constriction begins near the tip of the sac. Under both conditions the formed food-vacuole usually moves rapidly to the end of the oesophageal fibers.

normal conditions. The minor axis of the first one formed was about half as long as the diameter of the pharyngeal ring and that of the last one not more than one-sixth; whereas it usually is nearly twice as long in normal food-vacuoles. In their formation the oesophageal sac enlarged slightly, then a constriction appeared near its apex and soon a small portion of the sac was pinched off (Fig. 4 $A_1 - E_1$). This passed rapidly to the posterior end of the body then almost immediately rounded up, after which it moved slowly, decreased rapidly in size and seemed to disappear entirely. There were no visible particles in any of these food-vacuoles.

Ciliary activity in the pharynx was seen in nearly all the compressed campanellae examined, but food-vacuoles formed in only a small percentage of them. In all but a few of these the formation of food-vacuoles ceased immediately after ciliary activity in the pharynx had ceased and in these few only one vacuole formed after this.

These results indicate that the enlargement of the oesophageal sac is dependent upon activity of the cilia in the pharynx but not upon activity of those on the peristome, and they show that the formation of the food-vacuole is not specifically dependent upon ciliary action in the pharynx or the size of the oesophaeal sac or the presence of particles in suspension in the fluid in it.

3. In a compressed specimen of *Campanella tincta* five small food-vacuoles were formed in succession and rapidly transported to the posterior end of the body; then there suddenly occurred a very violent upheaval in the cytoplasm, after which a large food-vacuole was formed and transported, but very slowly and only a short distance, after which it turned sharply, nearly stopped moving and soon rounded up. Two more large vacuoles were formed after this and these also moved slowly and only a short distance, then stopped and rounded up. The large food-vacuoles were more than 20 times as large as the small ones; they moved much more slowly than the small ones and not more than half as far before they stopped and rounded up.[1]

Similar results were obtained in observations on several other compressed specimens. In one of these a very large food-vacuole formed, slowly moved back a short distance, turned sharply in its course, rounded up and stopped. Then a very small vacuole formed and moved rapidly, past the large one, nearly to the posterior end of the body after which it moved very slowly and rounded up. This was followed by the formation of two more small vacuoles, both of which moved rapidly past the large one to the posterior end of the body (Fig. 5). The large vacuole was closely observed under the oil-immersion objective. It did not move appreciably but rapidly decreased in size and disappeared entirely in three minutes.

The fact that some of these food-vacuoles went only about one-fourth as far as others before their rate of speed rapidly decreased cannot be understood on the assumption that they passed through a tube and were propelled by peristalsis in it. It can however, be readily understood on the assumption, postulated above, that their movement was due to the action of fibers which can move freely and are not fixed in their spacial interrelationship.

4. A specimen of *Vorticella similis* was mounted in tap-water and the oesophageal sac measured at maximum size. Then the tap-water was replaced by distilled

[1] The junior author asserts that in his observations on the effect of various chemicals on the size of the food-vacuoles in *Vorticella similis*, he frequently saw very small "needle-like" vacuoles form.

water and the sac measured again, after which the distilled water was replaced by 0.006 M lactose in distilled water and the sac measured once more. The averages obtained for the minor axis under these three conditions were respectively 10.5μ, 11.5μ and 12.5μ.

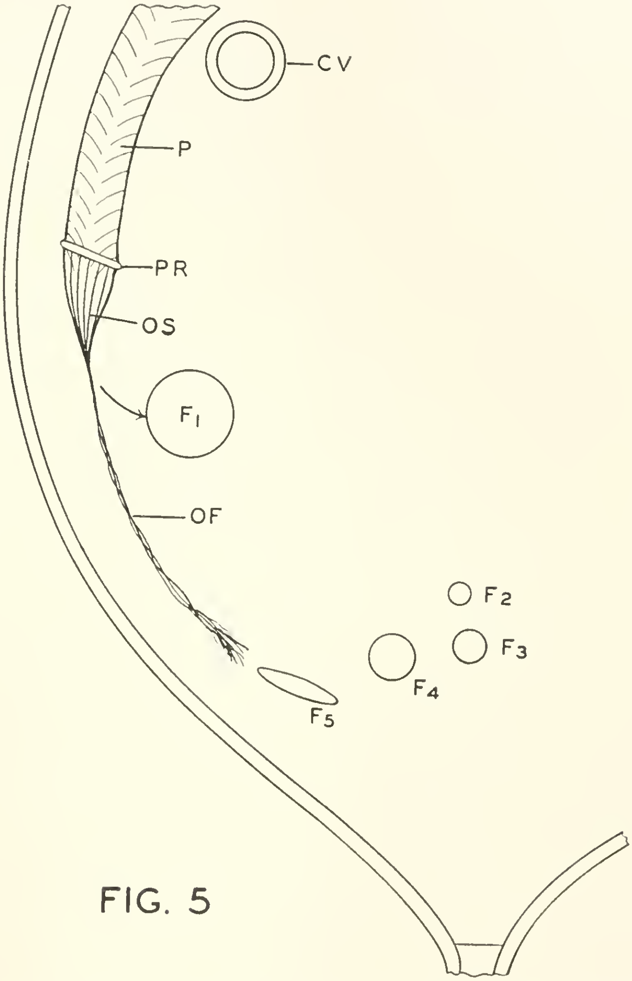

FIG. 5

FIGURE 5. Camera outline of a portion of a compressed specimen of *Campanella tincta,* showing differences in the size of successively formed food-vacuoles and difference in their direction and extent of movement.

CV, contractile vacuole; *P,* pharynx; *PR,* pharyngeal ring; *OS,* oesophageal sac; *OF,* oesophageal fibers; $F_1 - F_5$, *five* food-vacuoles formed in the order given; arrow, direction of movement.

Note that the first vacuole in this series was very much larger than the rest and moved only a short distance before it rounded up and that the four succeeding small vacuoles passed the large one and moved much further before they stopped and became spherical. F_1 was drawn very soon after it has been formed, $F_2 - F_5$ immediately after F_5 had been formed, i.e. after F_2 and F_3 had decreased considerably in size.

During one of the measurements of the oesophageal sac in the lactose solution a food-vacuole in the adjoining cytoplasm suddenly fused with the sac and caused a very marked increase in its size, immediately after which a portion of it was constricted off as an abnormally large food-vacuole. Immediately before the fusion took place the minor axis was 12.5μ long and during fusion it increased to 16.5μ.

The fact that the food-vacuoles in the cytoplasm can fuse with the oesophageal sac, strongly supports the conclusions reached above, namely that there is nothing in the nature of an oesophageal tube in these organisms and that the rapid movement of the food-vacuoles after they leave the pharynx is due to the action of a mobile structure which does not have a fixed position in the cytoplasm and does not prevent direct contact between the vacuoles and the cytoplasm.

Discussion

Greeff (1870) long ago observed that the food-vacuoles in *Campanella umbellaria* pass from the pharynx toward the posterior end of the body much more rapidly than the adjoining cytoplasm and he concluded consequently that they are not carried by the cytoplasm. He maintains, as stated above, that there is a long tube ("der Oesophagus") which extends into the cytoplasm from a spindle-shaped structure ("der Trichter") at one end of the pharynx. He asserts that in the "Trichter" the food which has been forced into it by the cilia in the pharynx, is formed into small spindle-shaped masses, which pass rapidly through the oesophagus" into the cytoplasm and that a membrane then forms at the surface of each mass and thus produces a food-vacuole. He accounts for the rapid movement of the food-vacuoles by assuming that they are forced through the "Oesophagus" by waves of contraction in it, i.e. by peristalsis.

Kahl (1935, p. 652) confirms Greeff in reference to the "oesophageal" tube in *Campanella* and concludes that such a tube is present in all the peritricha. He says: "Der Ösophagus ist bisher meist übersehen worden; er scheint aber nach eigenen Untersuchungen nie zu fehlen, ist aber nur bei grösseren Arten gut erkennenbar."

The evidence presented above indicates that Greeff is correct in his contention that at the end of the pharynx there is a funnel-shaped structure to which is attached a long narrow structure which extends into the cytoplasm, but it indicates that the latter is a bundle of fibers instead of a tube and that the former is a sac, a portion of which is separated off to form a food-vacuole, rather than a funnel in which the food is formed into spindle-shaped masses which become food-vacuoles after they have been transported through the tube into the cytoplasm. It also indicates that the food-vacuoles are propelled from the pharynx to the posterior end of the body by the action of fibers, not by contraction in the wall of a tube.

Kitching (1938, p. 87) recently observed the rapid movement of the food-vacuoles referred to above, in a considerable number of species in several genera but he found no evidence of an oesophageal tube in any of them. He concludes that the rapid movement of the vacuoles is due to waves of contraction, in accord with Greeff's contention, but that the contraction is in the cytoplasm, not in the wall of a tube in it. He says: "It is concluded that the food-vacuoles are propelled over the determined course [i.e. from the pharynx to the posterior end of the body] by contractions in the surrounding protoplasm."

It is obvious, however, that to propel a vacuole by contraction in surrounding protoplasm which is not fixed as it is in a tube, the viscosity of the protoplasm would have to be continuously lower in front of the vacuole than back of the contracting region. There is no evidence indicating that this obtains. Kitching's hypothesis consequently has no objective support.

Nirenstein (1905), Gelei (1934) and others maintain that in *Paramecium* the food-vacuole is separated from the pharynx by the pressure of protoplasmic currents. Bütschli (1889, p. 1405) and Bragg (1935, 1936) contend that contraction of the distal end of the pharynx is also involved. Lund (1941) holds that neither is involved and that the vacuole is separated from the pharynx by the action of fibers which are attached to the pharynx and extend for a considerable distance into the cytoplasm. These views will be considered in a later paper.

The Initiation of the Constriction of the Food-vacuole from the Pharynx

It is generally assumed that the initiation of the constriction of the food-vacuoles from the pharynx is correlated with the size of the enlargement at the end of the pharynx. For example, Hall and Nigrelli (1930) referring to *Vorticella* say: "After the basal portion of the gullet reaches a certain size, it is rapidly constricted from the rest of the gullet and then separated completely as a food vacuole." The fact, however, that (as demonstrated above in observations on *Campanella*) successively formed food-vacuoles sometimes vary enormously in size, shows that the initiation of their separation from the pharynx is only very superficially correlated with their size, if at all.

Bozler (1924) maintains that in *Paramecium* solid particles are necessary for the formation of food-vacuoles and that such particles must come in contact with the membrane at the end of the pharynx before a food-vacuole begins to form. Bragg (1935) maintains that while contact of a large particle with the inner surface of the "vacuolar membrane" always causes immediate separation of the food-vacuole from the pharynx, it is not necessary. We have, in observations on *Campanella* and *Vorticella,* repeatedly seen food-vacuoles form which contained no visible particles and we have seen some of these vacuoles disappear in the cytoplasm so rapidly that very little, if any, digestion could have occurred. These facts seem to show that these vacuoles contained no solid particles, and consequently that solid particles were not involved in their formation. Moreover, Schewiakoff (1891) and Wallengren (1901) assert that they observed food-vacuoles form in solutions which were free from solids.

Kitching (1938) observed that if *Pyxidinium aselli* is mounted in "1/16 to 1/8% agar" food-vacuoles form without ciliary action on the "disc" or in the "gullet." We have confirmed this in observations on Campanella. We also observed that there is no change in the size of the pharynx during the separation of the food-vacuoles from it. This separation is therefore not correlated with changes in ciliary action in the pharynx or with contraction in it.

It will be demonstrated presently that the size of the food-vacuoles depends upon the chemical composition of the surrounding medium. This seems to show that the chemical composition of the solution in the food-vacuoles has something to do with their separation from the pharynx, but it in no way accounts for the enormous variation in size referred to above, which occurred with no variation in the surrounding medium.

What is it, then, that sets off the process which separates the food-vacuoles from the pharynx?

It is highly probable that waves start at fairly regular intervals in the pharyngeal ring and pass simultaneously down all the oesophageal fibers and that each of these sets of waves initiates a constriction in the oesophageal sac, if it contains sufficient fluid to make a constriction possible. If this is true, the size of the vacuole is correlated with the rate at which fluid is forced into the oesophageal sac by the cilia in the pharynx and the rate at which it leaves this sac by osmosis. If these processes and the interval between successive waves depend upon the composition of the surrounding fluid, the temperature and the physiological state of the organism, it accounts for the observed variation in the size of the food-vacuoles and the intervals between their formation. If the food-vacuoles are separated from the pharynx by waves in the oesophageal fibers, one would, moreover, expect to find the observed correlation between the location of the constriction on the oesophageal sac and the size of the vacuole and also the observed absence of a constriction when the sac is very small. There would still remain, however, the problem of the origin of the periodic waves.

Food and Feeding

The observations considered in this and the following sections were made on *Vorticella similis* as follows:

Several small pieces of substance with vorticellae attached were mounted in pond-water or culture-fluid between two parallel ridges of vaseline on a slide. A cover-glass was then added and pressed down until the pieces of substance were much flattened, but not enough to interfere with the activities of the vorticellae. In such preparations the fluid could readily be changed as desired by applying a strip of filter paper to one edge of the cover-glass, and if the flow of fluid was continued so as to provide sufficient oxygen any selected vorticella could be studied under low or high magnification as long as desired and the effect of various substances on its activities ascertained.

Vorticella feeds almost exclusively on bacteria, but all sorts of particles in suspension in the surrounding fluid are carried into the vestibulum in the currents produced by the peristomal cilia. Many of these are, however, immediately carried out again in the outgoing current produced by the cilia in one region of the vestibulum. Nearly all the rest and some gelatinous substance secreted by the peristome or the walls of the feeding apparatus, are forced through the pharynx into the oesophageal sac by the pharyngeal cilia.[2] There is, however, great variation in the kind of particles that are selected and ingested by different individuals in the same preparation and by the same individual at different times. Yeast-cells, e.g. are, at any given time, freely ingested by some individuals and rigidly rejected by others, and freely ingested by a given individual at one time and rigidly rejected at another.

It is well known that when the food-vacuole leaves the pharynx the concentration of particles in the fluid in it is usually very much greater than it is in the fluid which enters the vestibulum. Greeff (1870) maintains that the cilia in the pharynx come in direct contact with the particles in it and force them through the fluid into the oesophageal sac and that consequently only a relatively small amount of water is carried in with the particles. Nirenstein (1905) and Bozler (1924) referring to *Paramecium* maintain that the pharyngeal cilia force almost nothing but fluid into the oesophageal sac until it has become nearly maximum in size and then almost nothing but solid particles until it is well filled with them. Both of these views would account logically for the relatively great concentration of solid particles in the newly formed food-vacuoles. However, the results of our extensive and detailed observations do not confirm either of them.

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[2] In *Vorticella* mounted in distilled water or in lactose (0.05 M) in tap-water or in solutions of NaCl, this gelatinous substance is very evident. It gelates as the vacuoles decrease to minimum in size (probably owing to the increase in acidity) and then solates as they increase in size. It is highly probable that it is formed under all conditions, as it appears to be in *Folliculina*, judging from the results of observations made by Andrews (1923).

We found that when the oesophageal sac begins to enlarge the concentration of solid particles in it is usually only slightly greater than in the fluid which enters the vestibulum, but that as the sac enlarges, the concentration of particles in it usually increases greatly.

Selective action of the pharyngeal cilia would account for the concentration of particles in the newly formed food-vacuoles, but it would not account for the observed gradual increase in concentration in the oesophageal sac except on the assumption of gradual increase in selective ciliary action. This is, however, not at all probable. How then can the gradual increase in concentration be explained?

It will be demonstrated presently that after the food-vacuole is formed fluid usually leaves it rapidly, owing to difference in osmotic concentration of the internal and external fluids. It is consequently practically certain that fluid passes continuously from the oesophageal sac out into the cytoplasm as it enlarges. The increase in the concentration of the particles in the fluid in the oesophageal sac is therefore, in all probability, due to this loss of fluid. Moreover, Frisch (1937), in observations on *Paramecium*, has demonstrated that fluid passes from the pharynx into the adjoining cytoplasm. If this obtains in *Vorticella*, it accounts for the probable increase in the concentration of solid particles as the fluid in which they are suspended passes through the pharynx.

The junior author, in his measurements of the food-vacuoles in *Vorticella* in different solutions, repeatedly saw the oesophageal sac suddenly decrease in size and at times, especially in distilled water, alternately decrease and increase like "the pumping of a heart." In one specimen in 0.014 M NaCl the oesophageal sac gradually increased to 11.56μ in diameter, then suddenly decreased to 8.84μ in diameter, then remained without further measurable change in size for 20 seconds and then left the pharynx. The decrease in size observed under these conditions was, however, doubtless due to the forcing of fluid from the oesophageal sac back into the pharynx, probably by pressure on the surface of the sac by the action of the oesophageal fibers.

The Size of the Food-vacuoles and the Time Required for their Formation

Introduction

No detailed measurements have heretofore been made on the size of the food-vacuoles in the peritricha or the time required for their formation. The results reported indicate, however, that while there is much variation in different individuals under the same conditions and in the same individual under different conditions, consecutive vacuoles do not vary much either in size or in the time required for their formation. Hall and Nigrelli (1930) imply, e.g. that in

Vorticella sp. the food-vacuoles are fairly uniform in size and the "intervals" between their formation rather constant for a given individual.

We measured many food-vacuoles and the time required for them to form in *Vorticella similis* in various solutions. Some of the results obtained will be considered in the following paragraphs. A more extended account of the work will be presented in a subsequent paper by the junior author.

Methods

Several vorticellae attached to a fragment of *Lemna,* or to a short hair, were mounted in a drop of water between two parallel ridges of vaseline on a slide and covered with a cover-glass. The slide was then put on the mechanical stage of the microscope and a narrow strip of filter paper, long enough to reach over the edge of the stage, placed at one edge of the cover-glass between the ridges of vaseline. Then some of the solution to be tested was placed on the slide at the other edge of the cover-glass between the ridges of vaseline and more added as, owing to the action of the filter paper, it flowed through under the cover-glass. A specimen which extended from its attachment well out into the current of solution was now observed. After the vorticella had been subjected to this current for ten minutes and thoroughly adapted to the new solution, measurements under an oil-immersion objective were made by means of a stopwatch and an ocular micrometer, on a series of successive food-vacuoles, in reference to the time required for their formation and their maximum size, i.e. the length of the minor axis, as they were about to leave the pharynx. Another solution was then passed through under the cover-glass for ten minutes, after which measurements were made on another series of successive food-vacuoles in the same specimen or in a different specimen in the same solution. This was repeated with still other solutions. Then the whole process was repeated with other specimens. The results obtained are presented in Tables I and II.

TABLE I

Time required to form food-vacuoles in Vorticella similis

	Time in seconds required to form each of seven consecutive vacuoles in each of six individuals, selected at random					
	In pond-water			In distilled water		
	a	b	c	d	e	f
	40	48	52	69	79	42
	38	56	51	70	83	58
	51	56	29	50	83	58
	62	50	39	67	86	46
	31	50	39	75	56	54
	39	49	38	43	58	44
	42	46	39	60	?	35
Average	43.3	50.7	41	62	74.1	48.1
Total average	45			61.4		

Results

Table I shows that there was marked variation in the time required for the formation of consecutive food-vacuoles in all six vorticellae studied, that the time required varied much with the individuals under both conditions and that it was on the average much longer in distilled than in pond-water. The results presented demonstrate, therefore, that the rate of formation of food-vacuoles is much higher in pond-water than in distilled water.

Table II shows that the successive food-vacuoles in each of the five specimens tested varied greatly in size in all the solutions used, but that the food-vacuoles in different specimens in the same solution and in the same specimen in different solutions varied even more. It shows that in the four individuals measured the average length of the minor axis of the vacuoles ranged from 7.3 to 15.2μ in the solution of lactose, from 4 to 11μ in the solution of NaCl and from 5.4 to 7.3μ in the solution of $CaCl_2$. It indicates that the vacuoles were on an average slightly larger in pond-water than in distilled water, the same in size in redistilled water and the solution of lactose, much smaller in the solution of NaCl, and the smallest in the solution of $CaCl_2$.

TABLE II

Variation in the size of the food-vacuoles in Vorticella similis *and the effect of various substances on its size*

A, a specimen subjected successively to distilled and pond-water; a, b, c and d, four specimens, each subjected successively to the solutions indicated. The lactose, NaCl and $CaCl_2$ solutions were made with redistilled water and they were equal in osmotic concentration.

All the measurements for each specimen in a given solution were made on successive vacuoles.

	Length in micra of minor axis at maximum size															
	Distilled water	Pond-water	Redistilled water				Lactose 0.026 M				NaCl 0.014 M				$CaCl_2$ 0 01 M	
Designation of specimens.....	A	A	a	b	c	d	a	b	c	d	a	b	c	d	a	b
No. of vacuoles measured......	5	5	8	9	9	9	8	9	9	5	9	6	4	6	5	5
Minimum.......	12.7	13	11.56	7.14	7.46	8.5	13.6	7.48	6.12	8.84	6.8	5.44	3.4	4.76	6.8	4.76
Maximum.......	14.1	16	17	10.2	9.18	10.2	17	10.1	8.16	10.88	14.16	7.48	4.76	5.44	8.8	6.12
Average........	13.4	14.1	14.6	8.3	8.2	9.9	15.2	8.7	7.3	9.9	11	6.3	4	5.2	7.3	5.4
Total average for a and b....			11.26				11.75				9.12				6.35	
Total average....			10.12				10.13				7.36					

Discussion

The osmotic concentration of the solution of lactose used was obviously much higher than that of the redistilled water. The fact that the food-vacuoles formed in these two fluids were practically the same in size indicates, therefore, that osmotic concentration is not involved in regulating the size of the vacuoles.

The solutions of lactose, sodium chloride, and calcium chloride used were equal in osmotic concentration and in acidity. The differences in the size of the food-vacuoles formed in these solutions were therefore not correlated with either of these two factors. They consequently must have been correlated with the chemical properties of the substances in the solutions.

The hydrogen-ion concentration of the distilled water used was pH 5.5 and that of the pond-water pH 8.2; the osmotic concentration of the latter was much higher than that of the former and they differed greatly in chemical composition. The results referred to above indicate that the size of the food-vacuoles is not specifically correlated with the osmotic concentration or the acidity of their contents. The difference in the size of the vacuoles observed in pond-water and distilled water was therefore not due to either of these two factors. It consequently must have been due to difference in the chemical composition of their contents.

The results in hand seem to show therefore that the size of the food-vacuoles in *Vorticella* is largely, if not entirely, dependent upon the nature of the chemicals they contain.

As stated above the rate of formation of food-vacuoles is higher and the vacuoles are larger in pond-water than in distilled water. The rate of ingestion of fluid is therefore higher in the former than in the latter, but since these fluids differ greatly in acidity, osmotic concentration, and chemical composition, the difference in the rate of ingestion may be due to any one or any combination of these factors.

We are well aware that some of the results presented in this section are equivocal, and that more results are needed before valid conclusions concerning the regulation of the size of the food-vacuoles and the rate of ingestion can be reached. We had intended to extend the observations made and to investigate the effects of other chemicals in various concentrations, but other duties interfered and we see no prospect of continuing the work in the near future. We are therefore presenting these inadequate results with the hope of encouraging further work.

CHANGES IN THE SIZE AND THE FORM OF THE FOOD-VACUOLES

Introduction

After the food-vacuoles have been separated from the pharynx they move rapidly to the posterior end of the body on a definite course, as previously stated, then slowly on a very indefinite course to the vestibulum. They are spindle-shaped until they reach the posterior end of the body then they usually become spherical and gradually decrease in size to a minimum, remain so for about two minutes and then rapidly increase in size again (Fig. 6).[3] Numerous measurements were made with a stopwatch and an ocular micrometer on the time required for these changes and their extent. The following results were obtained:

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[3] During the decrease in size the particles in suspension frequently, but not always, aggregate near the center of the vacuole, leaving a clear space at the surface (Fig. 6), which soon disappears, but usually forms again when the vacuole begins to enlarge, after which the particles soon become equally distributed.

Change in form.—The time required for the change in the form of the food-vacuoles from spindle-shaped to spherical varies enormously. Under some conditions it occurs almost immediately after the vacuoles have reached the posterior end of the body. Under others it requires an hour or more and under still others it probably does not occur at all.

The rate of change in form seems to be closely correlated with the viscosity of the content of the vacuoles. The particles in suspension in the fluid in the vacuoles which changed rapidly in form were invariably in violent Brownian movement, indicating low viscosity, whereas those in the vacuoles which changed slowly were often practically stationary, indicating high viscosity. In specimens which had ingested carmine granules or lactose (0.025–0.05 M) the change was consistently very slow.

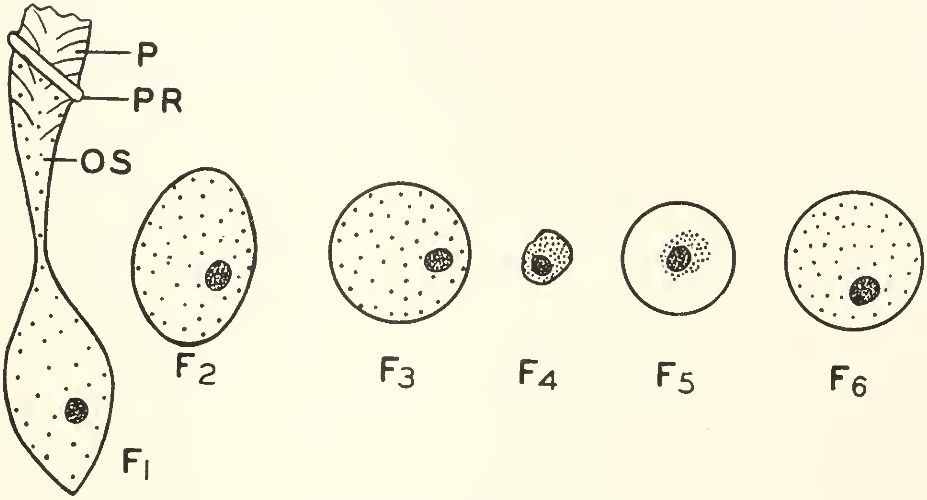

Figure 6. Camera outlines showing the separation of a food-vacuole from the pharynx in *Vorticella similis* and subsequent changes in its size and form.
P, pharynx; *PR*, pharyngeal ring; *OS*, oesophageal sac; F_1, a food-vacuole in the process of separation from the oesophageal sac; F_2–F_6, subsequent stages in the food-vacuole.

Decrease in size.—The decrease in size is due to loss of fluid. This ordinarily continues until there is no perceptible fluid left in the vacuoles and the surface membrane is in close contact with the mass of particles. Consequently, if the vacuoles contain relatively few particles, they decrease much more than if they contain many, and if the particles are large, yeast-cells, e.g. the surface becomes very irregular. Many of the vacuoles which were measured decreased three-fourths in diameter, i.e. to one sixty-fourth in volume. Under some conditions there is, however, still considerable fluid in the vacuoles when they have become minimum in size and under these conditions the shrinkage is obviously less. The reduction in size requires from one to three minutes.

The loss of fluid, resulting in the decrease in the size, is probably due in part to difference in the osmotic concentration of fluids in the vacuoles and the cytoplasm (that of the latter being higher than that of the former)[4] and in part to inward pressure of the elastic membrane on the surface of the vacuoles, which was stretched by the pressure of fluid forced into them by the pharyngeal cilia.

If the decrease in the size of the food-vacuoles is correlated with excess external osmotic concentration, the extent of change in size should decrease if the internal osmotic concentration is increased. This can readily be accomplished by adding physiologically neutral osmotic substance to the surrounding medium.

Observations were consequently made on vorticellae in pond-water containing lactose in various concentrations, and the vacuoles formed in each concentration measured at short intervals after they left the pharynx. It was found that no vacuoles were formed in concentrations higher than 0.05 M, that in concentrations of 0.05 M and lower the vacuoles decreased in size and that they decreased least in the highest of these, i.e. 0.05 M, but that the decrease in this concentration was definitely less than in pond-water, the maximum being not more than one-third in diameter in place of three-fourths or more. It was also found that after the vacuoles had reached minimum size, those formed in the lactose solutions contained much more fluid than those formed in pond-water and were never irregular in shape, like many of those formed in pond-water.

These results show that difference between internal and external osmotic concentration is involved in the observed decrease in the size of the vacuole and they indicate that the inward pressure of the membrane around them is also involved.

If this is true, it is obvious that fluid leaves the vacuoles, not only from the time they reach the posterior end of the body until they have become minimum in size, but continuously from the very beginning of their formation, for these two factors function in the oesophageal sac as well as in the vacuole, since the vacuole is, as previously demonstrated, merely a portion of the sac.

In vorticellae in the lactose-pond-water solution, it was repeatedly observed that the food-vacuoles often coalesce with each other after the sudden increase in size and that owing to this and lack of elimination of undigested substance, the body became well filled with huge vacuoles. Coalescence of vacuoles was not observed in pond-water or culture fluid. The lactose must consequently produce changes in the vacuolar membrane which make it possible.

Increase in size.—The vacuoles usually remain minimum in size for nearly two minutes, then *very rapidly* increase until they are nearly, if not quite, as large as they were originally, after which they remain fairly constant in size until their content is discharged into the vestibulum (Fig. 3).

The increase in size requires on an average, a little less than three seconds. During this time the vacuole is literally flooded with fluid from the cytoplasm. This fluid usually first appears as a well defined layer between the membrane and the viscous central mass, then this mass disintegrates and the solid particles in it soon become uniformly dispersed with violent Brownian movement throughout. Digestive enzymes are doubtless carried from the cytoplasm into the vacuoles with the fluid that enters, for digestion begins soon after the vacuoles have enlarged.

In *Paramecium* and some other ciliates numerous so-called neutral red bodies aggregate on the surface of the food-vacuoles. It is maintained by some that these bodies contain digestive enzymes, that they enter the vacuoles and are therefore involved in digestion. In the Peritricha there is no aggregation of such bodies on the food-vacuoles and there is no indication that any enter. They are consequently in all probability not involved in digestion in these organisms.

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[4] The osmotic concentration of the fluid in the cytoplasm, as will be demonstrated in the last section of this paper, is approximately 0.3 atmospheres higher than that of the surrounding medium.

The inflow of fluid, resulting in increase in size, is probably entirely due to greater osmotic concentration within the vacuole than without. If this is true, the internal osmotic concentration must increase greatly during the time that the vacuole remains minimum in size. This could readily be brought about by transformation in the vacuole of osmotically inactive to osmotically active substance, for example, starch to sugar.

In food-vacuoles which contain lactose, the gelatinous substance in them, referred to above, increases greatly in viscosity as the vacuoles decrease in size (as indicated by observations on Brownian movement) and then decreases greatly as they increase in size. The increase in viscosity is correlated with increase in acidity (as will be demonstrated presently). It may well be that this increase in acidity causes chemical changes in the gelatinous substance which result in increase in osmotic concentration and that this in turn causes the rapid inflow of fluid from the cytoplasm which in turn, owing to decrease in acidity, causes the observed decrease in viscosity.

Changes in the Hydrogen-ion Concentration in the Food-vacuoles in Vorticella

Introduction

Numerous observations have been made by several investigators on the hydrogen-ion concentration in the food-vacuoles in various protozoa. Nearly all the results obtained indicate that as the food-vacuoles pass through the body the hydrogen-ion concentration first increases, then decreases and then remains nearly constant. However, only a few of the observations concern the extent of these changes. Shapiro (1927) on the basis of changes in the color of indicator dyes, concludes that in the food-vacuoles in *Paramecium* the hydrogen-ion concentration increases to pH 4, then decreases to pH 7, that in *Vorticella* it increases to pH 4.5, then decreases to pH 7 and that in *Stylonichia* it increases to pH 4.8, then decreases to pH 7. Claff et al. (1941) using essentially the same methods conclude that in *Bresslaua* it increases to between pH 4.2 and 3 and then decreases (extent not given). Mast (1942) using similar methods and others found that in *Amoeba* it increases to pH 5.6, then decreases to pH 7.3. And Howland (1928) on the basis of results obtained by injecting dyes into the food-vacuoles, concludes that in *Actinosphaerium* it increases to pH 4.3 ± 0.1 and then decreases to between pH 5.4 and 7.

These conclusions indicate that the change in hydrogen-ion concentration in the food-vacuoles differs greatly in the protozoa. The validity of some of them is, however, so equivocal that further investigations are highly desirable. Detailed observations were therefore made on the changes in the hydrogen-ion concentration in the food-vacuoles in *Vorticella*. Two methods were used: one consisted of observations on the solubility of crystals in the vacuoles; the other of observations on changes in the color of ingested yeast-cells which had been stained with various indicator dyes.

Ingested crystals indicating acidity

Neutral red was added to pond-water (pH 8.2) and left for several hours. During this time numerous long needle-like yellowish brown crystals formed. Some of these crystals were broken up by mounting a little of the solution containing them under a cover-glass on a slide and vigorously tapping the cover-glass. Some of the broken crystals were then drawn under the cover-glass on a preparation containing several vorticellae in pond-water. The vorticellae occasionally ingested pieces of the crystals, some minute, others as long as the diameter of the vacuoles. A considerable number of vacuoles containing such pieces were carefully observed. No changes were seen in any of the pieces of the crystals until after the vacuoles which contained them had left the pharynx and had decreased considerably in size (but not to a minimum) then they suddenly dissolved.

The relation between the solubility of these crystals and the acidity of the solution surrounding them was ascertained by adding some to Clark buffer solutions differing in hydrogen-ion concentration and to Hahnert culture solutions containing different quantities of HCl. It was found that their solubility is closely correlated with the acidity of the solutions and that the lowest acidity in which they dissolve readily is approximately pH 5 in the buffer solutions and approximately pH 4 in the HCl solutions (Mast, 1942).

The results obtained in the observations on the crystals in the food-vacuoles indicate, therefore, that the hydrogen-ion concentration of the fluid in the food-vacuoles increased from pH 8.2 to about pH 5 as the size of the vacuole decreased. But since the crystals dissolved before the vacuoles had (as stated above) reached their minimum size, the maximum acidity of the fluid in them must have been higher than pH 5. The results obtained in the following observations confirm this contention.

Ingested indicator dyes showing maximum and minimum acidity in the food-vacuoles

Methods.—Yeast-cells were boiled in distilled water containing respectively the following indicator dyes: meta cresol purple (range pH 1.2–2.8 and 7.4–9), thymol blue (range pH 1.2–2.7 and 8–9.6) metanil yellow (range pH 1.2–2.8), benzopurpurin (range pH 1.2–4), dimethyl yellow (range pH 2.8–4.4), brom phenol blue (range pH 3–4.6), methyl orange (range pH 3.2–4.4), methyl red (range pH 4.2–6.3), brom cresol purple (range pH 5.2–6.8), congo red (range pH 3–5), brom thymol blue (range pH 6–7.6), neutral red (range pH 6.8–8), phenol red (range pH 6.8–8.4), nile blue (range pH 7.2–8.6), and cresol red (range pH 7.2–8.8). The yeast-cells stained well in benzopurpurin, brom phenol blue, congo red, brom thymol blue, neutral red and nile blue but not in any of the others.

Some yeast-cells stained with each of these six different dyes were put respectively into pond-water (pH 8.2) and presented to vorticellae in pond-water under cover-glasses.

In nearly all the preparations the vorticellae ingested some of the stained yeast cells, and in these they sometimes ingested them so freely that the food-vacuoles became well filled with them. The number in the vacuole could, however, be controlled by regulating the number in suspension in the surrounding medium.

Observations were made on numerous vacuoles, containing various numbers of yeast-cells, from the time the cells entered the oesophageal sac until they were discharged.

A series of Clark buffers[5] in small test-tubes was arranged in a row in a test-tube rack for each dye and an appropriate amount of the dye added to each buffer in the series. The acidity of adjoining buffers differed by 0.2 pH. The color of the stained yeast-cells in the vacuoles was, as the vacuoles formed and circulated in the body, continuously compared with those of the buffers in the series containing the dye under consideration and the hydrogen-ion concentration of that which it most nearly matched noted. It is assumed that this was the hydrogen-ion concentration of the substance in the vacuole at the time the comparison was made.

Congo red (pH 3, orange–pH 5, blue)

The results obtained with yeast-cells stained with congo red are more clear-cut than those obtained with any of the other dyes used. This is due to the brilliance and density of the color of the yeast-cells stained with this dye and to the striking change in color correlated with changes in hydrogen-ion concentration.

The yeast-cells in the pond-water in which the vorticellae were mounted were dense brilliant orange in color. Those which were ingested retained this color for an average of 75 seconds after the food-vacuole had left the pharynx, then, as the vacuoles decreased in size, they gradually became purple, then more and more bluish until the vacuoles had become minimum in size and the cells, if there were but a few in a vacuole, sky-blue in color (about pH 3). This color they now retained for an average of nearly 2 minutes, i.e. until the vacuoles very rapidly increased in size, then the cells suddenly became orange of the same shade as that which they had when they entered the vacuoles. This color was retained until the content of the vacuoles was discharged which usually occurred within half an hour. There was no indication of digestion in the discharged yeast-cells. A typical record taken from our notes reads as follows:

(2:10 p.m.) A yeast-cell entered a vacuole; (40 sec. later) the vacuole, containing only one yeast-cell, left the pharynx, spindle-shaped, 12μ long and 8μ wide, yeast-cell still orange; (75 sec. later) yeast-cell slightly purple,[6] vacuole spherical, 8μ in diameter; (75 sec. later) yeast-cell sky-blue (about pH 3), vacuole 3μ in diameter, slightly irregular in form; (2 min. later) yeast-cell orange, vacuole spherical, 8μ in diameter; (15 min. later) yeast-cell orange, no change in structure, vacuole same in size.

The results presented indicate, therefore, that the acidity of the fluid in the food-vacuoles in *Vorticella* increases nearly, if not quite, to pH 3 and that this is closely correlated with decrease in the size of the vacuoles.

The conclusion that increase in the acidity of the content of the food-vacuoles is closely correlated with decrease in size is strongly supported by results obtained in observations on congo red-stained yeast-cells ingested in 0.05 M lactose in pond-water. In these observations it was found that the food-vacuoles do not decrease as much in size as they do in pond-water without lactose, there always being considerable fluid left in them and that the color of the yeast-cells usually changes from orange to purple but never to blue, indicating that the acidity of the content of the vacuoles increases only to pH 5 in place of nearly to pH 3.

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[5] The following buffers were used: phthalate, pH 2.6–3.4; acetate, pH 3.6–5.6; phosphate, pH 5.8–8; borate, pH 7.8–10.

[6] Under high power (oil-immersion objective), it could be seen clearly that the central portion of the cells was still orange and that the purple was confined to a thin layer at the surface.

We repeatedly observed that if the food-vacuoles contained many congo red-stained yeast-cells, the cells did not become blue. We consequently made extensive observations on the relation between the number of yeast-cells in a vacuole and the extent of change of color in them and found the following:

In the vacuoles which contained five cells or fewer there usually was a change in color from orange to blue, but it required considerable longer in those which contained five than in those which contained only one or two cells. In those which contained six to nine cells, there usually was a change from orange to purple but not to blue, and the decrease in the size of the vacuoles was much less than in those which contained only a few cells. In the vacuoles which contained ten or more yeast-cells no change in color was observed and there was but little if any decrease in size. Two typical records from our notes follow:

(10:05 a.m.) A vacuole containing five yeast-cells left the pharynx; (2 min. later) yeast-cells getting purple, vacuole but little larger than the five cells; (1 min. later) cells bluish; (15 sec. later) cells blue, very little fluid in vacuole, irregular in form; (30 sec. later) cells turning orange, vacuole clearly larger, nearly spherical, hyaline layer at surface; (45 sec. later) cells orange, vacuole spherical, original size.

(10:30 a.m.) A vacuole containing about ten yeast-cells left the pharynx; observed continuously for six min.; no perceptible change in the color of the yeast-cells or the size of the vacuole.

These results show that the extent of change in color from orange toward blue in congo red-stained yeast-cells in the food-vacuoles and the extent of decrease in size of the vacuoles vary inversely with the number of yeast-cells in the vacuoles. They consequently support the conclusion that increase in the acidity of the content of the vacuoles is correlated with decrease in their size.

The question now arises as to whether or not the extent of change in color depends upon the time that the yeast-cells are in the vacuoles. Information concerning this question was obtained by making observations on cells which entered the vacuole at different times. Since the formation of the vacuole required from 30 to 60 seconds, this sometimes differs by nearly 60 seconds. It was repeatedly observed, however, that if a yeast-cell enters immediately after the vacuole begins to form it takes just as long for it to become purple after the vacuole has left the pharynx as it does if the cell enters just before the vacuole leaves it. Moreover, several vacuoles were studied in which one cell had entered at the beginning of formation and another just before the end of formation, and it was found that in all these vacuoles the two cells became purple and blue at the same time, although one of them had been in the vacuole nearly 60 seconds longer than the other. These results show that the acidity of the solution in the forming vacuoles is not high enough to have any perceptible effect on the color of yeast-cells and that the observed changes in color in them is not specifically correlated with the time they have been in the vacuoles.

Brom phenol blue (pH 3, yellow–pH 4.6, blue; benzopurpurin pH 1.2, violet–pH 4, red)

The results obtained with brom phenol blue confirm in general those obtained with congo red. With vorticellae in pond-water the stained yeast-cells were dense sky-blue when they entered the food-vacuoles and they became distinctly greenish yellow when the vacuoles had reached minimum size, but their color corresponded more nearly with buffer, pH 3.2 than pH 3. When the vacuoles increased in size the yeast-cells rapidly became blue again. The results obtained with brom phenol blue therefore indicate that the maximum acidity reached by the substance in the vacuoles is pH 3.2, i.e. not quite so high as is indicated by those obtained with congo red.

The yeast-cells stained with benzopurpurin were deep red when they entered the food-vacuoles and no appreciable change occurred as the vacuoles passed through the body. If the maximum acidity in the food-vacuoles is actually pH 3 as the results obtained with congo red indicate, one might expect some evidence of change in the color of the cells stained with this dye, for its range extends from pH 1.2 to 4. The difference in color between buffer pH 3 and pH 4 was however so inconspicuous that it would be extremely difficult to distinguish in yeast-cells in food-vacuoles. The fact then that no change in color was observed in the food-vacuoles containing yeast-cells stained with benzopurpurin, does not seriously militate against the results obtained with congo red and brom phenol blue.

The results presented, therefore, seem to prove that the acidity of the substance in the food-vacuole in *Vorticella* increases from somewhat less than pH 5 to a maximum of pH 3.2 as the size of the vacuole decreases to a minimum and that the acidity very rapidly decreases as the size of the vacuole suddenly increases, and they show that this decrease extends beyond the highest limit of the ranges for the dyes used, namely pH 6.8, but they do not show how far beyond this range it extends. The results obtained with brom thymol blue and neutral red concern this, and also the acidity of the content of the oesophageal sac.

Brom thymol blue (pH 6, yellow–pH 7.6, blue); neutral red (pH 6.8, red–pH 8, amber); nile blue (pH 7.2, blue–pH 8.6, purple)

The yeast-cells stained with brom thymol blue were deep blue when they entered the vorticellae in pond-water. In the oesophageal sac, they became distinctly yellowish, pH 6.4, if there were but few present. After the vacuoles had formed and left the pharynx and began to decrease in size they soon became bright lemon yellow, pH 6; then when they suddenly increased in size they rapidly became yellowish blue, like buffer pH 6.8, possibly pH 7, but positively not so blue as pH 7.2 and not nearly so blue as they were when they entered the vacuoles. The results obtained with brom thymol blue consequently indicate that the minimum acidity reached is approximately pH 6.9.

The yeast-cells stained with neutral red were brownish yellow (pH 8.2) when they entered the vacuoles. They very soon became reddish pink after the vacuoles had left the pharynx and began to decrease in size, but there was no appreciable change in color when the vacuoles later suddenly increased in size. The color of the buffers in the prepared series was essentially the same from pH 5 to pH 7, but at pH 7.2 it was distinctly yellowish. There was no indication of this color in the yeast-cells in the old vacuoles. The acidity in these cells therefore did not decrease to pH 7.2. These results therefore support the conclusion reached on the basis of those obtained with brom thymol blue, namely, that the minimum hydrogen-ion concentration reached in the substance in the food-vacuoles in *Vorticella* during the process of digestion is between pH 6.8 and 7, i.e. that the substance in the food-vacuole decreases greatly in acidity but does not actually become alkaline.

The yeast-cells stained with nile blue in pond-water were sky-blue and there was no change in color in those which were ingested. These results therefore have no bearing on the problem under consideration.

It can be concluded, then, that in *Vorticella* after the food-vacuole leaves the pharynx, the acidity of its content increases from approximately pH 6.4 nearly to pH 3 in about two minutes, with a decrease in size during this time to about 1/27 of its original volume, that it then remains nearly constant in acidity and in size for nearly two minutes, after which it very rapidly increases in size with a very rapid decrease in acidity to about pH 6.9. The problem concerning the processes involved in the changes in size has been considered in a preceding section; that concerning those involved in the changes in acidity will be considered in the following section.

Discussion

Shipley and DeGaris (1925) maintain that in *Paramecium* the fluid in the food-vacuole first becomes alkaline, then acid, then alkaline again. We obtained no evidence whatever indicating a preliminary alkaline phase in the food-vacuoles of *Vorticella*. Shapiro (1927) also failed to find any indication of it in this genus, but he maintains that he found a preliminary alkaline phase in *Paramecium* if the culture fluid is neutral but not if it is alkaline. It would seem, however, that in alkaline solutions, as Howland (1928) has well said, "it obviously should have been more prominent than in neutral solutions." The contention of Shipley and DeGaris is consequently equivocal. Moreover, evidence will be presented in a subsequent paper which indicates that it is not valid.

The food-vacuoles in *Actinosphaerium* into which Howland (1928) injected dyes contained active ingested organisms. These, owing to metabolism, undoubtedly caused increase in the acidity of the fluid in the vacuoles and the mechanical injury produced by the pipet used in the process of injection also augmented the acidity. The maximum acidity she observed, namely pH 4.3 ± 0.1, is therefore higher than that which obtains under normal conditions in vacuoles which do not contain living organisms.

Claff et al. (1941) maintain that in culture fluid containing neutral red, the fluid in the food-vacuole in *Bresslaua* becomes pink and also the organisms in it after they die, indicating increase in acidity. They hold that this increase in acidity is due to "a sudden release of an acid into the newly-formed food-vacuole" from the surrounding cytoplasm. But they also maintain that there are numerous "cherry red granules" in the cytoplasm and that many of them aggregate on the surface of the vacuole. We have made many observations which strongly indicate that the pink color observed by Claff et al. in the fluid was due to the effect of the "cherry red granules" on the transmitted light, not to dye in the fluid, and that the pink color in the dead organisms was due to the acid produced in them as they died, not to acid in the fluid around them, for similar changes in color occur in organisms which die in neutral red solutions which are not in the food-vacoules.

To obtain accurate results with dyes concerning the hydrogen-ion concentration of the content of the food-vacuoles in protozoa, it is therefore necessary to avoid injuring the cytoplasm around the vacuoles and to consider the effect of colored granules in the cytoplasm on the light transmitted through it, and the acid produced by metabolism and death of organisms in the vacuoles.

In the methods used in the observations on changes in acidity in the food-vacuoles in *Vorticella* considered above all these sources of error were avoided. The results obtained must therefore be fairly accurate.

Factors Involved in the Changes in Acidity in the Food-vacuoles

It is widely held that change in acidity observed in the food-vacuoles in the protozoa is due to secretion of acid or base by the cytoplasm adjoining the vacuoles (Greenwood and Saunders, 1894; Nirenstein, 1905; Lund, 1914; Howland, 1928; Claff et al., 1941). Mast (1942) maintains, however, that this does not obtain in *Amoeba*. He says that in this organism "the cytoplasm secretes neither acid nor base" and he concludes (p. 203): "The increase in the acidity of the fluid in the food-vacuoles probably is due to respiration in the ingested organisms, chemical changes associated with their death, disintegration of the ingested plasmalemma, impermeability to acids of the membrane around the vacuoles and diffusion of fluid from the vacuoles. The decrease in acidity is due to diffusion of alkaline fluid from the cytoplasm into the vacuoles. The cytoplasm secretes neither acid nor base."

Let us consider these views in reference (1) to the increase and (2) to the decrease in acidity observed in the food-vacuoles in *Vorticella*.

(1) *Increase in acidity*

If the increase in acidity in the food-vacuole is due to secretion of acid by the surrounding cytoplasm, the acid must pass from the cytoplasm either into the oesophageal sac or the food-vacuoles. We have demonstrated that fluid passes continuously out of the food-vacuoles from the time they begin to form until they have become minimum in size, i.e. during the time that the acidity in them increases to maximum. Consequently, if the increase in acidity is due to secretion of acid by the cytoplasm, it must pass into the pharynx or the vacuole against an outward current of fluid. This is highly improbable. Moreover, since the acidity of the content of the food-vacuoles reaches pH 3.2 and that of the adjoining cytoplasm is, as will be demonstrated presently, approximately pH 7.4 the acid in the vacuoles could come from the cytoplasm only by active secretion. There is, however, no indication whatever of a structure by means of which this could be accomplished. Secretion of acid by the cytoplasm into the vacuole is therefore not at all probable.

If living organisms in the food-vacuoles are involved in the increase in acidity in them, there obviously should be no change in acidity in food-vacuoles which do not contain living organisms. The following observations concern this:

Vorticellae were mounted in normal pond-water, then this was replaced by sterile pond-water by letting it flow continuously through the preparation for at least five minutes, then yeast-cells stained with congo red in sterile pond-water were added. The vorticellae ingested some of the yeast-cells and the color of those ingested changed as the vacuoles proceeded on their course. No difference in these changes and those which occur in normal pond-water, either in time or shade, could be detected.

This experiment was repeated several times with sterile pond-water and also with distilled water. The results obtained agree with those presented above, with the exception that in distilled water it required a little less time for the change from orange to purple (increase in acidity) and the vacuoles were not quite so small when it occurred. This is doubtless due to the fact that the distilled water used was pH 5.5 and contained no buffers, whereas the pond-water was pH 8.2 and contained buffers, and therefore required more acid to produce the observed increase in acidity.

It can consequently be concluded that if metabolism in living organisms in the food-vacuoles in Vorticella is a factor in the production of the observed increase in acidity, it is of minor importance.

Yeast-cells which have been stained with congo red are, as previously stated, not digested. In the food-vacuoles formed by vorticellae in distilled water, containing these cells, there is consequently very little if any digestion. It was found, however, that the increase in acidity in these vacuoles is just as great as it is in those which contain an abundance of digestible substance. It is therefore obvious that digestion is not extensively, if at all, involved in the production of acid in the food-vacuoles. What, then, causes the observed increase in acidity in the food-vacuoles?

Lund (1914, p. 14) demonstrated that in *Bursaria* the acidity of the substance which enters the vestibulum increases as it passes thru the pharynx and he concluded that this shows that the cytoplasm secretes acid and pours it into the pharynx. There is, however, a more likely cause of the increase in acidity observed by Lund.

In the protozoa the cilia in the feeding apparatus are very active during the process of feeding and they perform a considerable amount of work in forcing fluid into the vestibulum and through the pharynx into the oesophageal sac. Metabolism in them and in the cytoplasm associated with them is, therefore, high. This, owing to the production of carbonic, lactic, and other acids, causes increases in the hydrogen-ion concentration[7] of the fluid as it passes through the feeding apparatus. After the fluid has entered the oesophageal sac, some of it passes out through the limiting membrane into the cytoplasm and still more after the food-vacuole has been formed and has left the pharynx, as shown by its rapid decrease in size. Moreover, Bozler (1924), Fortner (1924, 1926), Eisenberg (1925), Müller (1932) and especially Frisch (1937) have demonstrated fairly conclusively that fluid passes continuously from the pharynx into the cytoplasm during the process of feeding. This would further increase the acidity of the substance in the pharynx as it passes through, if the wall of the pharynx is impermeable to the acids produced by metabolism but permeable to bases, as it may well be. It is therefore highly probable that the increase in acidity in the pharynx observed by Lund is due to the end products of metabolism rather than to secretion by the cytoplasm.

The acid in the pharynx obviously passes into the food-vacuole, and if the membrane at the surface of the vacuole is impermeable to acids but permeable to bases, the acids will remain in the vacuole as fluids and bases pass out, and the acidity of its content will increase. Mast (1942) accounted for the increase in the acidity of the food-vacuole in *Amoeba* by means of similar assumptions. The source of the acid appears however to differ greatly in the two organisms.

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[7] By adding brom thymol blue to weakly buffered culture fluid containing protozoa, it can readily be demonstrated that they produce acid in the process of metabolism.

The question now arises as to whether the loss of fluid from the food-vacuole in *Vorticella* is great enough to produce the observed increase in acidity in it. We do not know precisely what the hydrogen-ion concentration of the content of the food-vacuole is when it leaves the pharynx, but the results obtained in observations on ingested yeast-cells stained with brom thymol blue indicate, as stated above, that it is about pH 6.4. However, Lund (1914) found in observations on *Bursaria* that ingested vitellin and yolk granules in an alkaline solution containing litmus, change from blue to red in the pharynx, before they enter the food-vacuole. This shows that the content of the forming food-vacuole in *Bursaria* is distinctly acid. Lund has reproduced the color assumed by the litmus-stained granules in the pharynx. By comparing this color with that of litmus paper in each of a series of buffers, ranging from pH 5.2 to pH 6.6, it was found that it is more nearly like the litmus paper in buffers pH 5.8 (and lower) than that in any of the other buffers in the series. This indicates that the solution ingested by *Bursaria* changed from distinctly alkaline approximately to pH 5.8. These results support the conclusion reached above, namely, that the hydrogen-ion concentration of fluid ingested by *Vorticella* increases considerably before the food-vacuole leaves the pharynx, and they indicate that it probably increases to pH 6. If this is true, the acidity of the food-vacuoles in *Vorticella* increases approximately from pH 6 to a maximum of pH 3.2 as the vacuoles decrease in size.

As previously stated, the decrease in the size of the food-vacuoles and the increase in the acidity of their content varies greatly, the one being roughly proportional to the other. Let us therefore consider the results obtained in actual measurements of the changes in size and acidity observed in a typical vacuole. These results show that the vacuole selected decreased in size from an ellipsoid $8 \times 12\mu$ to a sphere 3μ in diameter and that the acidity of its content increased approximately from pH 6 to pH 3.2. If it had been full of fluid at pH 6 when it was maximum in size, it would have contained 6×10^{-19} moles of H^+, and if it had been full of fluid at pH 3.2 when it was minimum in size, it would have contained 89×10^{-19} moles of H^+, i.e., it would have contained nearly 15 times as much H^+ when it was minimum in size as it would have when it was maximum in size. According to the postulated hypothesis it should contain the same amount. The loss of fluid during the reduction in size would therefore not have been sufficient to account for the observed increase in acidity on the basis of this hypothesis. The vacuole was however not full of fluid. It contained approximately three percent of solids when it was maximum in size and 97 percent when it was minimum. It therefore contained about three percent less than 6×10^{-19} moles of H^+ or 5.82×10^{-19} moles at maximum size, and 97 percent less than 89×10^{-19} moles of H^+ or 2.67×10^{-19} moles at minimum size, i.e., less than half as much as at maximum. The increase in the concentration of hydrogen-ions, owing to loss of water during the decrease in the size of the vacuole, would therefore seem to be ample to account for the observed increase in acidity if there is, in accord with our hypothesis, no loss in hydrogen-ions.

(2) Decrease in acidity

The decrease in acidity in the food-vacuoles is, as previously stated, accompanied by a very rapid and extensive inflow of fluid from the cytoplasm. The fact that this inflow requires only about three seconds and is many times as great in volume as the fluid already in the vacuole, indicates very strongly that the decrease in acidity is due to low acidity of the fluid which enters from the cytoplasm, and that nothing in the nature of secretion is involved.

THE HYDROGEN-ION CONCENTRATION OF THE CYTOPLASM IN VORTICELLA

No one has previously investigated the hydrogen-ion concentration of the cytoplasm in any of the ciliates but several have investigated it in the rhizopods. Pantin (1923) maintains that the hydrogen-ion concentration of the cytoplasm in a small marine amoeba is pH 7.6–7.8 in the plasmasol, pH 7.2 in the plasmagel and pH 6.8 in the protruding pseudopods. Needham and Needham (1925) conclude that in *Amoeba proteus* it is pH 7.6 throughout, and Chambers, Pollack and Hiller (1927) contend that in *Amoeba proteus* and *Amoeba dubia* it is pH 6.9 ± 0.1.

Mast (1942) has considered these contentions critically. He contends that the methods used are not reliable and comes to the conclusion on the basis of his own observations that the hydrogen-ion concentration of the cytoplasm in *Amoeba proteus* is approximately pH 7.4.

The results presented above show that in *Vorticella* the flooding of the food-vacuole with fluid from the cytoplasm usually causes the vacuole to increase about 25 times in volume and the acidity of their content to decrease approximately from pH 3.2 to pH 6.9. But since the vacuole contains approximately 97 percent solids at minimum size and only some five percent at maximum, the fluid in it increases more than 500 times. Since the two fluids mixed in the vacuole are buffered, and their relative amounts and the hydrogen-ion concentration of one of them and that of the mixture are known approximately, that of the other (the fluid in the cytoplasm) can be ascertained approximately, by mixing appropriate buffers in proper proportions and measuring the hydrogen-ion concentration of the mixture. This was done, and it was found that if one part of a pH 3.2 buffer is added to 500 parts of a pH 7 buffer (the approximate proportion of the two fluids mixed in the vacuole), the hydrogen-ion concentration of the mixture is pH 6.98. This indicates that the hydrogen-ion concentration of the cytoplasm in *Vorticella* is slightly higher than pH 7, i.e., considerably higher than that of the cytoplasm in *Amoeba proteus.*

THE FUNCTION OF THE CHANGES IN THE HYDROGEN-ION CONCENTRATION IN THE FOOD-VACUOLES

Hemmeter (1896), Howland (1928) and Claff et al. (1941) maintain that the increase in acidity in the food-vacuoles in protozoa serves to kill the ingested organisms. Nirenstein (1905) concludes, however, that in *Paramecium* the acidity of the content of the food-vacuoles does not become high enough to kill the ingested organisms and Mast (1942) comes to the same conclusion in reference to *Amoeba.* It is consequently doubtful whether the increase in acidity functions as a killing agent in any protozoa, and according to Greenwood and Saunders (1894), Nirenstein (1905) and Mast (1942) it does not function directly in digestion in *Amoeba* and *Paramecium,* for digestion does not begin in these organisms until after the acidity in the food-vacuoles has decreased to a minimum.

Vorticella, as previously stated, feeds almost exclusively on bacteria. After the bacteria have been carried into the forming food-vacuole by the action of the cilia in the feeding apparatus, they swim actively about in the fluid in it and they continue swimming until a few moments after the vacuole has left the pharynx and has decreased somewhat in size, then they stop abruptly (all coming to rest at practically the same instant) and usually soon aggregate in a dense mass in the central region of the vacuole. They have doubtless been killed, for they do not become active again when the vacuole enlarges and the mass breaks up. This also occurs if lactose (0.05 M) is added to the culture fluid.

The hydrogen-ion concentration of the fluid in the vacuoles when the bacteria became inactive, could not be accurately measured, but the results obtained in observations on ingested yeast-cells stained with congo red indicate that it is not higher than pH 5. Moreover, in culture fluid containing 0.05 M lactose the acidity, as stated above, increases only to approximately pH 5. The bacteria in it are, therefore, not subjected to higher concentration of acid than this.

The lethal concentration of acid for the bacteria was ascertained by adding to given quantities of culture fluid different quantities of hydrochloric acid and measuring the time the bacteria in the culture fluid lived. It was found that they lived indefinitely in the culture fluid at pH 5 and more than 30 seconds in the culture fluid at pH 4. It is consequently obvious that death of the bacteria in the food-vacuoles is certainly not entirely due to the increase in acidity.

The time between the separation of the vacuole from the pharynx and the cessation of movement of the bacteria in it, was measured with a stopwatch. It was found that this varies considerably in consecutive vacuoles in the same individual, but that the average for different individuals is fairly uniform. The variation for ten consecutive vacuoles in a typical individual was 14 to 18 seconds with an average of 16.3 seconds.

It requires, as stated above, about 50 seconds to form a food-vacuole. The bacteria which enter when it begins to form are, therefore, in it about 66 seconds before they are killed, whereas those which enter just before it leaves the pharynx are in it only about 16 seconds. The cause of death must therefore be due largely, if not entirely, to changes in the content of the vacuole after it leaves the pharynx. There are, as previously stated, two very prominent changes during this time, increase in acidity and decrease in fluid. It was demonstrated above that the increase in acidity is not fatal. Death in the food-vacuoles is therefore probably due to the loss of fluid. Mast (1942) comes to the same conclusion in reference to the cause of death in the food-vacuoles in *Amoeba.* He contends that the loss of fluid augments the decrease in oxygen in the vacuoles due to respiration in the bacteria, to such an extent that it is fatal.

There is no visible indication of digestion of the bacteria in the food-vacuoles until after the acidity in them has decreased to minimum. This seems to show that the increase in acidity does not function in digestion. There are however profound changes in the vacuole while the acidity in it is maximum for, as previously stated, the osmotic concentration of the fluid in it during this time increases greatly. It may well be, therefore, that the increase in acidity functions in the production of this increase in osmotic concentration, e.g. by hydrolizing complex molecules, which in turn functions in the inflow of fluid-carrying enzymes which facilitate digestion.

The decrease in acidity in the food-vacuoles is clearly correlated with digestion, but since it is merely the result of rapid inflow of fluid from the cytoplasm it is obviously not the result of anything in the nature of secretion by the cytoplasm.

The Osmotic Concentration of the Cytoplasm in Vorticella

If the decrease in the size of the food-vacuoles in *Vorticella* were entirely due to difference between internal and external osmotic concentration, and if the membrane at the surface of the food-vacuoles were permeable to water only, and if no osmotically active substance passes into the feeding apparatus from the cytoplasm, the osmotic concentration of the cytoplasm could be measured by changing that of the ingested fluid until there is no decrease in the size of the vacuole after it leaves the pharynx. It was however demonstrated above that inward pressure of the stretched membrane around the vacuole is functional in the decrease in its size, and it is highly probable that some osmotically active substance enters the feeding apparatus from the adjoining cytoplasm. The decrease in the size of the food-vacuole is therefore probably not closely correlated with the relation between the osmotic concentration of the fluid in the food-vacuole and that of the fluid in the cytoplasm. It was found however that the size of the entire body varies consistently with the osmotic concentration of the surrounding medium and that this relation can be fairly accurately measured. Observations on it were therefore made as follows:

A vorticella attached to a fragment of *Lemna* was mounted in pond-water or tap-water under a cover-glass supported on two parallel ridges of vaseline and the length and width of the body measured by means of an ocular micrometer. Then the water was replaced with a solution of lactose in pond-water or tap-water, left ten minutes and the vorticella again measured. This was now repeated with different concentrations of lactose and with different individuals. The results obtained in reference to length are presented in Table III. The width varied directly with the length, but it also varied with the surface viewed. It was therefore not recorded in the table.

Table III shows that the vorticellae decreased in size in the higher concentrations of lactose used, but not in the lower, and that the decrease varied directly with the concentration, but that it was greater in the vorticellae which had been adapted to pond-water than in those which had been adapted to tap-water. It shows that of the seven individuals adapted to pond-water, five became slightly smaller in 0.0125 M lactose in pond-water and two did not change in size, but that in 0.025 M lactose all became definitely smaller; whereas in the nine individuals adapted to tap-water only two became smaller in 0.0125 M lactose in tap-water and only seven became smaller in 0.025 M lactose. The lowest osmotic concentration which causes any decrease in size, is, therefore, a little lower than that of 0.0125 M lactose in pond-water for vorticellae adapted to pond-water, and a little higher than 0.0125 M lactose in tap-water for vorticellae adapted to tap-water. If, then, the decrease in size in the lactose solutions is due to the difference between internal and external osmotic concentration this difference must be slightly less than the osmotic concentration of 0.0125 M lactose for the vorticellae which have been adapted to pond-

Table III

Relation between the size of Vorticella *and the osmotic concentration of the surrounding medium*

Each specimen used was measured successively in the four concentrations; specimen a, three times in each; b, c and d, twice in each; and the rest once in each. The lengths of a, b, c and d given, are averages.

Length of body in micra				
Designation of specimens	Concentration of lactose in pond-water			
	0.05 M	0.025 M	0.0125 M	0 M
a	77.50	81.66	86.66	90.83
b	52.50	57.50	63.25	65.00
c	43.75	46.75	48.75	50.00
d	60.00	65.00	71.25	72.50
e	75.00	75.00	80.00	82.50
f	50.00	62.50	70.00	70.00
g	57.50	62.50	70.00	70.00
Total average	59.46	64.41	69.98	71.26
	Concentration of lactose in tap-water			
	0.05 M	0.025 M	0.0125 M	0 M
a_1	70.00	77.00	80.50	80.50
b_1	59.50	66.50	66.50	66.50
c_1	56.00	70.00	70.00	70.00
d_1	52.50	66.50	70.00	73.50
e_1	60.75	73.50	77.00	77.00
f_1	52.50	59.50	63.00	63.00
g_1	63.00	66.50	70.00	70.00
h_1	85.75	91.00	92.75	87.50
i_1	53.25	63.00	63.00	64.75
Total average	61.45	70.38	72.52	72.52

water and slightly more than that of 0.0125 M lactose for those which have been adapted to tap-water.

The osmotic concentration of the pond-water used, calculated from the depression of the freezing point, is at 22° C, equivalent to 0.79 atmospheres and that of the tap-water practically zero. The results presented indicate, therefore, that the lower the external osmotic concentration is, the greater the difference between internal and external osmotic concentration becomes.

The osmotic concentration of 0.0125 M lactose at 22° C is equivalent to 0.3282 atmospheres (International Critical Tables). That of the fluid in the cytoplasm must, therefore, be approximately equivalent to 0.3282 plus 0.79 atmospheres or 1.1 atmospheres in vorticellae adapted to pond-water, but only slightly higher than 0.3282 atmospheres in those adapted to tap-water.

Kitching (1938) concludes that in *Zoothamnium* sp., a freshwater peritrich, the excess of internal over external osmotic concentration is equivalent to that of 0.05 M sucrose, that is, four times as large as the results we obtained in our observations on *Vorticella*. This difference is much larger than would be expected in organisms which are so nearly alike in structure and habitat. Kitching's conclusion was based on results obtained with specimens treated with cyanide. It may well be, therefore, that it is not valid for specimens under normal conditions.

Mast and Fowler (1935) found that 0.005 M lactose in culture fluid is the lowest concentration which produces a consistent measurable decrease in the volume of *Amoeba proteus*. This indicates that the difference between internal and external osmotic concentration is much smaller in *Amoeba* than it is in *Vorticella*.

SUMMARY

1. The feeding apparatus in the Peritricha consists of a ciliated tube (the outer portion of which is called the vestibulum and the inner the pharynx) and about ten fibers (oesophageal fibers) which are attached to the distal end of the pharynx and extend as a bundle through the cytoplasm nearly to the posterior end of the body. There is no oesophageal tube.

2. The Peritricha feed largely on bacteria but various inanimate particles are also ingested.

3. At the end of the pharynx surrounded by the oesophageal fibers there is a cone-shaped sac (the oesophageal sac) which consists of a membrane probably produced by the interaction between the fluid in it and the cytoplasm around it.

4. The pharyngeal cilia force into the pharyngeal sac culture fluid with particles in suspension and usually gelatinous substance secreted by the peristome, the vestibulum and the pharynx.

5. The sac enlarges and becomes spindle-shaped. Then a portion of it is constricted off to form a food-vacuole.

6. The constriction is probably due to local simultaneous inward pressure of the oesophageal fibers.

7. The food-vacuoles vary greatly in size.

8. Initiation of the constriction in the sac and the size of the food-vacuole formed by it are not specifically correlated with the size of the sac or particles in suspension in the fluid in it or the chemical composition of this fluid, but they are to some extent dependent upon these factors, especially the chemical composition of the fluid.

9. The concentration of particles in suspension in the fluid in the oesophageal sac increases as the sac increases in size. This is largely due to the passage of fluid through the oesophageal membrane into the cytoplasm.

10. After the food-vacuole is formed, it passes rapidly on a fixed course through the cytoplasm to the posterior end of the body and then slowly on a varied course to the anal spot in the wall of the vestibulum. The rapid movement is probably due to waves passing synchronously along the fibers. The slow movement is due to cyclosis.

11. After the vacuole has reached the posterior end of the body it usually becomes spherical in form and gradually decreases greatly in size; as it decreases in size the acidity of its content increases to a maximum of pH 3.2, then it increases very rapidly in size and the acidity of its content decreases to pH 6.9.

12. The hydrogen-ion concentration of the fluid in the cytoplasm is approximately pH 7.

13. The decrease in size requires about two minutes. It is due in part to excessive external osmotic concentration and in part to inward pressure of the stretched membrane at the surface. The increase in size requires about three seconds. It is due to excessive internal osmotic concentration probably caused by chemical changes produced by the increase in the acidity of its content.

14. The increase in the acidity of the content of the vacuole is probably largely due to the production of acid, owing to metabolism in the peristome, the vestibulum and the pharynx and impermeability of the vacuolar membrane to organic acid, resulting in its retention and consequent concentration as the vacuole decreases in size.

15. The decrease in acidity is due to the flooding of the vacuole with fluid from the cytoplasm.

16. The osmotic concentration of the fluid in the cytoplasm of *Vorticella* varies directly with that of the surrounding medium. The former is higher than the latter approximately by an equivalent of 0.0125 M lactose or 0.3282 atmospheres.

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HYDROGEN-ION CONCENTRATION OF ALBUMEN AND YOLK OF THE DEVELOPING AVIAN EGG

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The changes in hydrogen-ion concentration of the albumen and yolk in the avian egg have been considered as indicative of the character of the metabolic processes occurring within the egg. These changes are, therefore, of importance in studies of embryonic development (Needham, 1931; Romanoff and Hayward, 1943).

A review of the literature (Needham, 1931; Romanoff and Romanoff, 1929) indicates that considerable work has been done on the hydrogen-ion concentration of the hen's egg and very little on the eggs of other species (Shklyer, 1937). It is of interest to know whether or not the changes in this physical property of eggs are similar in different species of birds.

METHODS AND MATERIALS

To obtain these data the present study includes the eggs of the Leghorn chicken (*Gallus gallus*), Ring-necked pheasant (*Phasianus torquatus*), Bobwhite-quail (*Colinus virginianus*), White Holland turkey (*Meleagris gallopavo*), Pekin duck (*Anas platyrhynchos*) and of domestic goose (*Anser anser*). Particular effort was made to obtain eggs as fresh as possible. On an average the chicken eggs were one or two hours old, while the age of the eggs of other species varied from 24 to 36 hours. The pH value was determined electrometrically, using an hydrogen electrode. The observations were carried out: (1) on the albumen until it was merged with the yolk sac, thus losing its physical entity, and (2) on the yolk until hatching time.

EXPERIMENTAL RESULTS

Egg albumen

The data for hydrogen-ion changes in albumen (Fig. 1 *A*) show a striking similarity in all the curves. The initial rise in pH, from as low as 7.6 to as high as 9.5, at the beginning of incubation is followed first by a rapid, then by a more gradual decrease to approximate neutrality. All values obtained were for the middle dense layer of albumen, for it has been shown (Romanoff, 1943a) that the pH values of the different layers do not vary to any great extent even in fresh eggs. The results presented here agree with those of Shklyer (1937) for hens, turkeys, ducks and geese in all essentials except for the initial pH values which were higher than ours. Evidently the eggs used by Shklyer in his experiments were of more advanced age before their setting for incubation.

Egg yolk

Previous observations show that at certain stages of incubation there is a morphological differentiation of egg yolk into two fractions—dense and liquefied (Romanoff, 1943b), with quite distinct electrical conductivities (Romanoff and Grover, 1936). For that reason, as was anticipated, the dense egg yolk undergoes an entirely different change in hydrogen-ion concentration. There is a gradual rise from slight acidity, of about pH 6.0, to an alkalinity of about pH 7.8 near the end of the incubation period (Fig. 1 *B*). Then the pH values decrease slightly before the time of hatching. The data for all species follow the same general trend of change. This again is in close agreement with data published by Shklyer (1937).

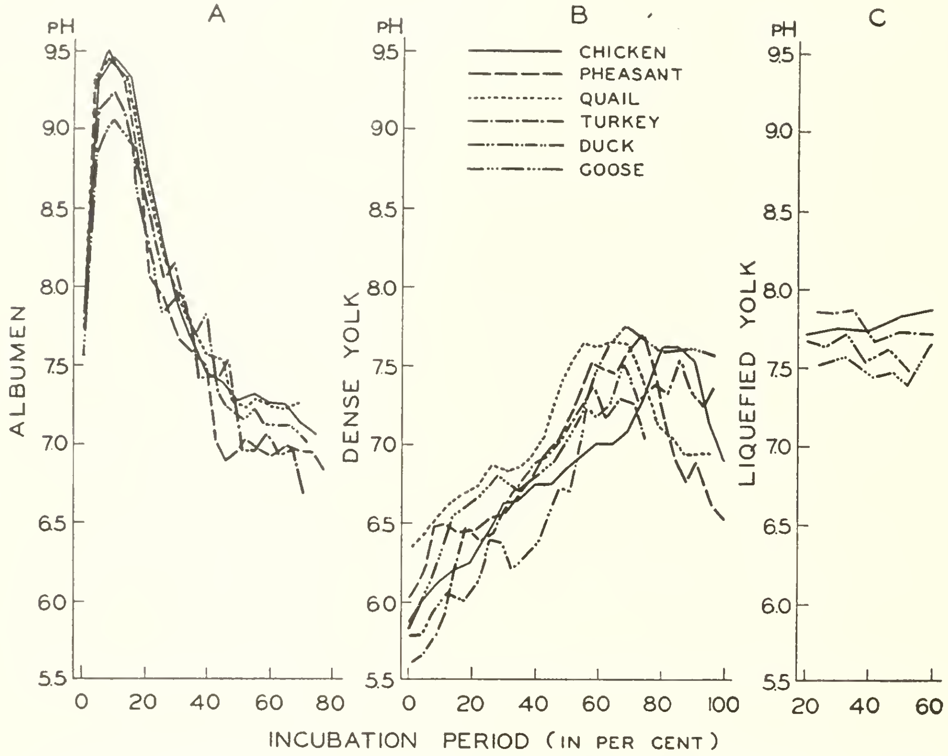

Figure 1. Changes in pH of avian eggs during embryonic development: *A*, albumen, *B*, dense yolk, and *C*, liquefied yolk. The data based on observations of over 600 eggs, daily averaging from 3 to 15 eggs for each species.

The liquefied yolk of avian eggs is consistently more alkaline in reaction than the semi-solid or dense portion (Fig. 1 *C*). According to Shklyer (1937) the liquefied yolk in the egg of the domestic fowl maintains an average pH value of about 7.7 throughout its period of existence.

Discussion

It is now recognized that to obtain comparable results, certain variables must be controlled in ascertaining changes in the hydrogen-ion concentration of the developing egg; this is especially true of the albumen. Two of the most important of these variables are the age of the egg at the time of setting for incubation, and the conditions under which they have been kept in storage.

The pH value of the albumen increases rapidly in an egg with aging (Romanoff and Romanoff, 1929). Unless eggs are set for incubation immediately after laying, the initial rise in pH of albumen during the development may not be fully observed. For this reason the values for the incubated eggs given by other investigators (see reviews by Romanoff and Romanoff, 1929; Needham, 1931; and Shklyer, 1937) frequently show high initial pH value, which afterwards has only a steady decrease towards acidity. The older the egg at the beginning of the incubation the nearer the pH value will be to the peak of alkalinity in the initial stages. In eggs kept under ordinary environmental conditions (temperature about 12–13° C.) for only about seven days, the portion of the curve showing a rapid rise in pH would be almost completely eliminated. Consequently without adequate control of the age of the egg, the results of many former studies demonstrate either only a very slight rise in the curve (Gueylard and Portier, 1925; Penionschkevitch, 1934; Berenstein and Penionschkevich, 1935) or none at all (Aggazzotti, 1913; Buytendijk and Woerdeman, 1927; Shklyer, 1937).

The initial rapid rise in the pH of the albumen during early incubation, as well as in storage, has been shown to be caused by the loss of carbon dioxide (Sharp and Powell, 1931; Brooks and Pace, 1938). It has been determined experimentally that the pH of the egg albumen is in direct relationship to the concentration of carbon dioxide in the incubator (Romanoff and Romanoff, 1930, 1933). With 10 per cent of carbon dioxide in the air the pH value of albumen does not rise at all—the curve flattens out, and the normal peak of high alkalinity is not observed. Similarly in storage at low temperature (0° C.), the rise in pH may be prevented by high carbon dioxide pressure (Moran, 1937).

It is the author's experience that with the eggs of the same preincubation age, the incubating temperature, within the range of embryonic survival, 35.5–39.5° C. (Romanoff, Smith and Sullivan, 1938), has a very insignificant effect on the variation in hydrogen-ion concentration of albumen and yolk. Also, negative results were obtained with the changes of relative humidity in the incubator (Penionschkevitch, 1934). However, according to Sharp and Powell (1931), the rise in pH of egg albumen prior to incubation is hastened by a higher temperature.

SUMMARY

The observations on incubated eggs of chicken, pheasant, quail, turkey, duck, and goose clearly indicate that changes in hydrogen-ion concentration of albumen, and of dense and liquefied portions of yolk are similar for all species studied, and suggest a pattern which may be characteristic of all avian eggs. In the albumen there is a rapid rise in pH, then a fall; in the dense yolk, a gradual rise with a slight fall at hatching; and in the liquefied yolk during its existence, high pH value without change.

The initial pH value of egg albumen during embryonic development depends chiefly upon the preincubation age of the egg—fresh eggs would give low, while older eggs would give high pH values of albumen at the beginning of incubation.

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ON THE INTERPRETATION OF RATES OF REGENERATION IN TUBULARIA, AND THE SIGNIFICANCE OF THE INDEPENDENCE OF MASS AND TIME[1]

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In his investigations into the metabolic basis of dominance in Tubularia stems and into the factors involved in hydranth regeneration, Barth (1938a, b) proposed as a measure of regeneration rate L/t, in which L is the length of the regenerating primordium and t the time in hours from the removal of the old hydranth to the appearance of a constriction between the primordium of the new hydranth and the rest of the stem. In some cases Barth also used $\pi r^2 L/t$, where r is the radius of the stem; but with uniform short stems r is virtually constant and L becomes an adequate estimate of the mass or volume of tissue involved. Although admitting that variation in primordium length occurs, Child (1940) criticized Barth's definition on the grounds that since growth or increase in cell number is not involved in the reconstitution, the inclusion of mass or volume in the measurement of rate is of doubtful validity. Child therefore maintained that $1/t$ gives a better indication of the kinetics of the process. Miller (1942), contending that Barth's definition is ". . . based on the implied assumption that length and time are inversely related to one another," also questioned the validity of the "implied assumption" on the basis of experiments in which length varied although time did not. Needless to say, Barth's definition of regeneration rate no more implies an inverse relation between the components of the ratio than the usual definition of velocity implies an inverse relation between distance and time.

It is important to note that $1/t$, commonly used as a measure of rate, is not free of ambiguity. Since the "one" in the numerator is a dimensionless quantity, it is clear that the magnitude defined by $1/t$ is not a rate in the generally accepted sense. Ordinarily other dimensions such as mass or length are involved, either alone or in combination, in determination of rate.[2] This is particularly true when such dimensions enter into the process being studied. Regeneration and differentiation, for example, while not usually accompanied by a net mass increase (growth), do imply the occurrence of transformation of mass from one type into another. Presumably these transformations can ultimately be referred to the formation of certain types of compounds at the expense of others. On this basis, then, a rational definition of rate of development would consider the mass of tissue transformed, and might be formulated in terms of mass per unit time. It is admitted that in many cases the difficulty of obtaining mass or volume measure-

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[1] Aided by grants from a Rockefeller Foundation fund administered by Dr. H. B. Steinbach; and from the Dyckman Fund of the Department of Zoology, Columbia University.

[2] The familiar exception is angular velocity, which has the form $1/t$. Even here however the "one" in the numerator is a consequence of the definition of an angle, which is the ratio of two lengths and therefore dimensionless.

ments of the differentiated tissue has made the use of the $1/t$ definition of rate necessary; nevertheless it may be of value to recognize that $1/t$ does differ from other rates, and to attempt a more precise interpretation of its significance. An analysis of this kind becomes of paramount importance in those investigations which seek to correlate metabolic and developmental rates. Such physiological rates as Q_{O_2}, Q_{CO_2}, or $Q^N_{O_2}$ involve a measurement of mass as well as time. Uncritical comparisons between these rates and $1/t$, which neglect the mass transformed, may well lead to erroneous conclusions.

In a previous study of the effects of various respiratory inhibitors on regeneration and respiration rates, the present authors (Moog and Spiegelman, 1942) used Barth's definition of regeneration rate in establishing that with certain inhibitors (e.g., urethanes, azide) rather drastic decreases in regeneration rates can occur without any concomitant measureable effects on Q_{O_2}. But subsequent experiments by Moog (1942) and Spiegelman (1942) indicated clearly that length and time may be independently affected, and so brought out the inadequacy of L/t expression of regeneration rate as a means of comparing directly the results obtained with a variety of agents. A similar situation was noted by Miller (1942), and also by Spiegelman and Goldin (1944) in interpreting the parallel effects of pH variation on regeneration and respiration rates.

Thus it seemed desirable to re-examine the extent of the independence of L and t under different experimental conditions in a more systematic way than had previously been attempted. On the basis of the data obtained and presented here, the significance of L/t as a rate measure will be examined, and the independent variation of L and t will be interpreted in terms of synthetic reactions in open systems approaching the steady state.

Materials and Methods

The solutions used were made up fresh each week in filtered sea water, and when necessary were adjusted to pH 8.2 with hydrochloric acid. Young unbranched stems uniform in translucence, length, and diameter were selected from colonies freshly gathered from the waters of Vineyard Sound or Cape Cod Bay during the months of July and August. Stem segments 6 mm. in length were cut from regions about five mm. proximal to the hydranth. Groups of 25 stem segments were kept in 100 ml. of the appropriate solution in partly filled, tightly stoppered flasks which were shaken at intervals to redistribute the oxygen. Solutions were changed daily, but the stems were kept in the flasks until they reconstituted or were finally transferred to fresh sea water, after four or five days. They were counted as totally inhibited, with rate of regeneration zero, if after being transferred they developed hydranths. In the temperature experiments, the desired temperatures, held constant to 0.5° or better, were obtained with water baths or incubators placed in cold rooms. Solutions in which stem segments were placed were brought to temperature before use.

Results

A. Narcotics

Table I summarizes the results obtained with different narcotics at various concentrations. In the case of ethyl urethane decreases are not observed in re-

TABLE I

The effects of narcotics on regeneration of Tubularia

Concentration (moles/liter)	Number of stems	Time (hours)	Rate		Length		Length/time	
			$1/t$	% of control	Micra	% of control	L/t	% of control
Ethyl urethane								
1. Control	23	38.1	0.0262	100.0	908	100.0	23.8	100.0
1×10^{-3}	24	37.0	0.0271	104.0	958	104.0	25.9	108.0
5×10^{-3}	23	38.8	0.0257	98.0	957	104.0	24.7	104.0
8×10^{-3}	19	39.6	0.0253	96.5	957	104.0	24.2	102.0
1×10^{-2}	22	49.7	0.0201	76.7	928	102.0	18.6	77.8
2×10^{-2}	22	56.9	0.0170	65.0	989	109.0	17.4	72.8
8×10^{-2}	25	60.7	0.0165	64.0	1019	112.0	16.8	70.0
2. Control	17	39.8	0.0251	100.0	963	100.0	24.2	100.0
5×10^{-3}	15	38.8	0.0257	102.0	1009	105.0	26.0	107.0
1×10^{-2}	18	38.6	0.0259	103.0	989	103.0	25.6	103.0
2×10^{-2}	17	52.8	0.0189	75.2	922	95.6	17.5	72.0
3×10^{-2}	11	59.3	0.0168	67.0	1016	106.0	17.1	70.5
4×10^{-2}	10	71.5	0.0140	55.7	981	102.0	13.8	57.0
3. Control	17	43.1	0.0232	100.0	949	100.0	22.0	100.0
1×10^{-7}	18	48.4	0.0207	89.0	1038	110.0	21.4	97.3
1×10^{-6}	18	44.8	0.0225	96.9	972	102.0	21.6	98.0
1×10^{-5}	17	45.4	0.0221	95.1	986	103.0	21.7	98.9
1×10^{-4}	19	49.5	0.0202	87.0	974	102.0	19.7	89.9
1×10^{-3}	18	44.5	0.0226	97.3	1045	110.0	23.6	108.0
1×10^{-2}	14	49.1	0.0204	87.8	888	93.5	18.1	82.1
5×10^{-2}	9	75.0	0.0137	59.0	764	80.5	10.2	46.5
Phenyl urethane								
4. Control	17	59.6	0.0168	100.0	812	100.0	13.8	100.0
1×10^{-4}	25	46.7	0.0214	127.0	773	94.1	16.5	120.0
1×10^{-3}	20	48.9	0.0205	122.0	914	111.0	18.7	133.0
1×10^{-2}	16	57.7	0.0173	103.0	884	107.0	15.3	111.0
2×10^{-2}	20	63.4	0.0158	94.1	858	105.0	13.5	98.0
3×10^{-2}	15	67.6	0.0148	88.0	767	93.5	11.3	82.0
4×10^{-2}	16	68.3	0.0147	87.5	800	97.5	11.7	84.9
5×10^{-2}	16	68.6	0.0146	87.0	757	92.1	11.0	79.8
5. Control	20	34.8	0.0288	100.0	1009	100.0	28.9	100.0
1×10^{-3}	17	30.3	0.0333	116.0	1100	109.0	36.3	122.0
1×10^{-2}	18	33.8	0.0296	103.0	963	95.4	28.2	98.0
2.5×10^{-2}	21	37.7	0.0266	92.4	822	81.5	21.8	75.0
4×10^{-2}	15	51.4	0.0195	67.6	851	84.2	16.6	57.3
5×10^{-2}	14	53.8	0.0186	64.6	536	53.1	10.0	34.5
6.5×10^{-2}	14	66.2	0.0151	52.4	380	37.6	5.7	19.7
Chloretone								
6. Control	24	26.6	0.0376	100.0	1150	100.0	43.2	100.0
1×10^{-2}	14	53.5	0.0187	49.6	920	80.0	17.2	39.9
1.25×10^{-2}	17	43.0	0.0233	61.9	1002	88.0	23.1	53.4
1.50×10^{-2}	18	46.0	0.0217	57.6	1006	88.0	21.7	50.2
1.75×10^{-2}	24	48.3	0.0207	55.0	878	76.3	18.1	41.9
2×10^{-2}	11	45.7	0.0219	58.1	935	81.3	20.4	47.1
2.25×10^{-2}	6	112.0	0.0089	23.6	560	48.7	5.0	11.6
2.50×10^{-2}	11	100.0	0.0100	26.5	590	51.2	5.9	13.7

generation rate (L/t) until the concentration reaches about 0.1 molar. Although the sensitivity does vary from group to group it is evident that the major effect is on the time to constriction. This is made strikingly apparent by figure 1, which is a plot of both primordium length and time to constriction, expressed as per cent of control, against the logarithm of the molar concentration multiplied by 10^6. Here over a concentration range which produces a 44 per cent decrease in the $1/t$ factor, the lengths of the regenerating primordia remain unaffected.

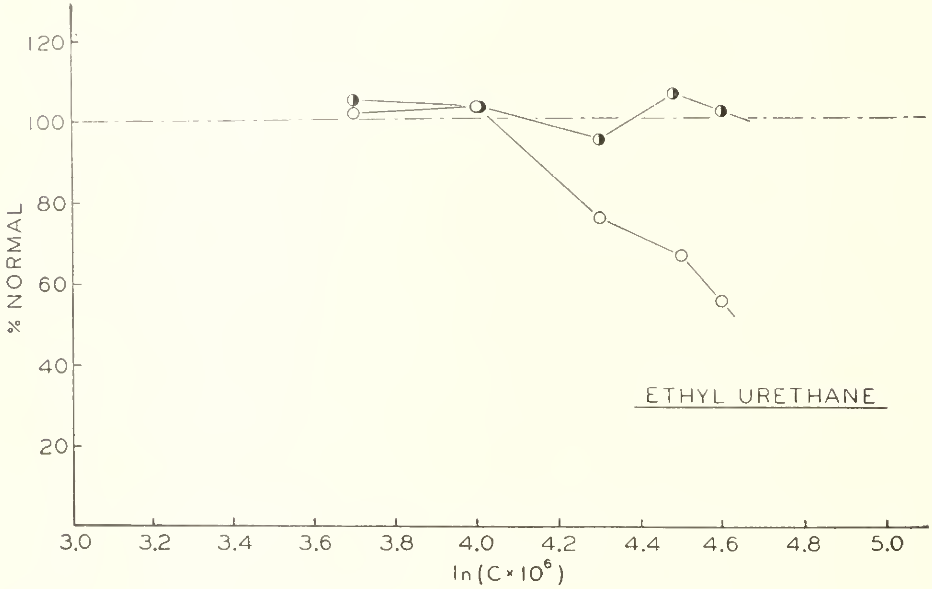

FIGURE 1. The effect of ethyl urethane on rate ($1/t$) of regeneration (open circles) and on length of the regenerating primordium (half-closed circles). Data from experiment 2, Table I.

With both phenyl urethane and chloretone the results are quite different. It is evident from Table I that within the concentration range in which decreases in regeneration rate are obtained, the inhibition involves comparable decreases in both the L and $1/t$ factors. The difference between the data obtained with these two narcotics and that obtained with ethyl urethane is illustrated by figure 2, which represents the data of experiment 6, with chloretone. In comparison with the effects of ethyl urethane, the parallelism of effects here is clear.

B. Cyanide, azide, and oxygen tension.

Tables II and III summarize the data obtained with these reagents. The regeneration rate L/t is extremely sensitive to even relatively low concentrations of cyanide. Thus 6×10^{-6} molar cyanide caused a 17 per cent reduction in L/t, and 5×10^{-5} molar a 61 per cent reduction. However it will be noted that these reductions were due almost entirely to diminishing $1/t$ values. This is illustrated by figure 3, which is a plot of the data of experiment 8; there it may be seen that in a concentration range which yielded a 61 per cent decrease in the $1/t$ factor the length was affected only to the extent of six per cent. The use of higher concentrations however led quickly to drastic reductions in the amount of tissue transformed.

In the case of azide in the range from 1×10^{-6} molar to 2×10^{-3} molar, the differential effect on the factors of the rate was not as clear-cut as in the case of cyanide. There was again however a tendency for the length to be less sensitive to lower concentrations than $1/t$. Thus in experiment 10, 7×10^{-4} molar azide decreased $1/t$ by 43 per cent and length by eight per cent. In experiment 12, the same concentration resulted in a 38 per cent decrease in $1/t$ and a 12 per cent decrease in length.

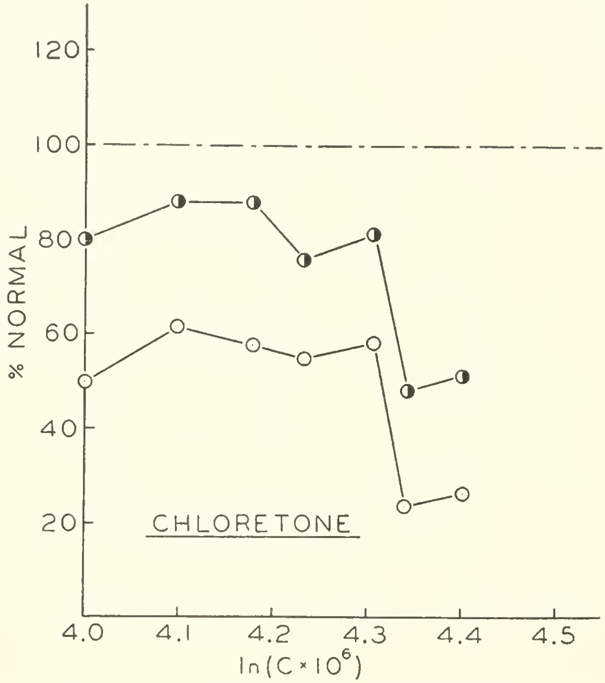

Figure 2. The effect of chloretone on rate ($1/t$) of regeneration (open circles), and on length of the regenerating primordium (half-closed circles). Data from experiment 6, Table I.

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Both cyanide and azide presumably act by poisoning the cytochrome-cytochrome oxidase system. A study of their effects on both respiration and reconstitution (Moog and Spiegelman, 1942) has however indicated that they follow different pathways in depressing the regeneration process, for cyanide inhibition of reconstitution was always accompanied by a strong depression of the respiratory rate, whereas azide, in a concentration which invariably cuts the reconstitution rate by at least 80 per cent, scarcely altered the rate of oxygen uptake at all. The data presented in Table II further indicate that the ultimate effect of azide differs from that of cyanide. In the case of cyanide a 20 per cent decrease in length is accompanied by a 70 per cent increase in time to constriction, but a

TABLE II

The effects of cyanide and azide on regeneration of Tubularia

Concentration (moles/liter)	Number of stems	Time (hours)	Rate		Length		Length/time	
			1/*t*	% of control	Micra	% of control	*L*/*t*	% of control
Sodium cyanide								
7. Control	20	36.4	0.0275	100.0	1175	100.0	32.1	100.0
5×10^{-5}	16	62.9	0.0159	57.7	1025	87.1	16.3	50.7
6.5×10^{-5}	18	76.0	0.0132	48.0	1039	88.2	13.7	42.6
8×10^{-5}	19	88.6	0.0113	40.4	1105	94.0	12.5	39.0
9.5×10^{-5}	18	117.6	0.0085	30.9	953	81.0	8.1	25.2
1.2×10^{-4}	24	128.0	0.0078	28.4	567	48.3	4.4	13.7
8. Control	19	26.2	0.0382	100.0	1176	100.0	44.9	100.0
1×10^{-6}	19	27.1	0.0370	97.0	1193	102.0	44.1	98.3
6×10^{-6}	18	30.8	0.0325	85.1	1146	97.4	37.2	82.3
2×10^{-5}	16	37.2	0.0269	70.5	1170	99.4	31.4	70.0
5×10^{-5}	20	63.9	0.0157	41.1	1103	93.9	17.3	38.5
7.5×10^{-5}	17	123.6	0.0081	21.3	889	76.1	7.3	16.2
9×10^{-5}	13	168.4	0.0060	15.7	445	37.8	2.6	5.8
9.5×10^{-5}	12	172.2	0.0058	15.2	380	32.2	2.1	4.7
1×10^{-4}	20	209.5	0.0048	12.6	141	12.0	0.7	1.6
Sodium azide								
9. Control	19	46.4	0.0215	100.0	986	100.0	21.3	100.0
2×10^{-6}	18	37.4	0.0268	125.0	1003	102.0	26.8	126.0
7×10^{-6}	17	41.6	0.0241	112.0	1013	115.0	24.3	114.0
2×10^{-5}	13	43.7	0.0229	106.0	1013	102.0	23.0	108.0
7×10^{-5}	18	48.5	0.0206	96.1	948	96.0	19.5	91.5
2×10^{-4}	14	71.9	0.0139	65.0	803	81.9	11.2	52.6
7×10^{-4}	15	92.6	0.0109	50.8	710	72.0	7.7	36.2
10. Control	18	29.6	0.0338	100.0	1349	100.0	45.2	100.0
1×10^{-6}	17	34.0	0.0294	75.8	1200	89.0	35.3	78.0
1×10^{-5}	17	49.5	0.0201	51.8	1162	86.4	23.5	51.9
1×10^{-4}	17	31.8	0.0314	81.0	1280	95.0	40.2	88.9
4×10^{-4}	18	36.5	0.0274	70.5	1278	94.6	34.8	76.8
7×10^{-4}	17	45.1	0.0222	57.1	1244	92.3	24.4	53.9
9×10^{-4}	13	51.2	0.0195	50.1	1244	85.0	18.8	41.5
9.5×10^{-4}	18	55.9	0.0179	46.0	1029	76.2	15.3	33.8
1×10^{-3}	11	56.7	0.0177	46.0	1031	76.5	12.1	26.7
1.3×10^{-3}	10	62.1	0.0161	41.5	989	73.4	9.1	20.1
11. Control	19	36.6	0.0274	100.0	1138	100.0	31.8	100.0
5×10^{-5}	18	33.7	0.0298	108.0	1079	94.6	31.5	99.2
2×10^{-4}	18	34.8	0.0288	105.0	994	87.2	28.2	88.7
6×10^{-4}	18	40.6	0.0246	90.0	1035	91.0	25.7	80.9
9×10^{-4}	19	55.6	0.0180	65.5	909	79.7	16.3	51.3
1.5×10^{-3}	13	76.1	0.0132	48.1	669	58.7	11.4	35.8
12. Control	18	30.6	0.0328	100.0	1162	100.0	38.0	100.0
2.5×10^{-4}	17	38.6	0.0259	79.0	1121	96.1	29.8	80.4
4×10^{-4}	19	37.6	0.0266	81.0	1059	90.8	28.1	78.4
5.5×10^{-4}	20	41.4	0.0242	74.0	1042	89.5	25.2	70.4
7×10^{-4}	19	49.4	0.0203	62.1	1024	88.0	20.7	57.8
8.5×10^{-4}	19	44.6	0.0224	68.1	886	76.1	19.9	55.5
1×10^{-3}	17	45.7	0.0219	66.9	897	77.0	19.8	55.3
2×10^{-3}	12	62.9	0.0159	48.5	314	27.0	5.0	13.9

TABLE III

The effect of varying oxygen tensions on regeneration of Tubularia
(Data from Barth, 1938b)

Oxygen tension (cc./liter)	Number of stems	Time (hours)	Rate		Length		Length/time	
			$1/t$	% of control	Micra	% of control	L/t	% of control
13. Control*	20	26.2	0.0382	100	1370	100	52.3	100
2.4	20	36.1	0.0277	72	1072	78	29.9	52
3.2	20	28.1	0.0263	93	1284	94	45.8	83
4.1	20	26.8	0.0374	98	1370	100	51.1	93
4.8	20	26.3	0.0381	100	1365	99	52.0	99
8.2	20	24.5	0.0408	107	1640	120	67.0	128
11.3	20	24.6	0.0407	106	1809	132	73.5	140
14.3	20	24.1	0.0415	106	1840	134	76.5	146
16.5	20	23.7	0.0422	111	1846	135	77.9	148

* Dish open to air.

comparable decrease in length by azide poisoning yields only about a 35 per cent increase in time.

In considering the role the oxygen-utilizing system plays in regeneration, it is of interest to examine, from the point of view of this paper, Barth's (1938b)

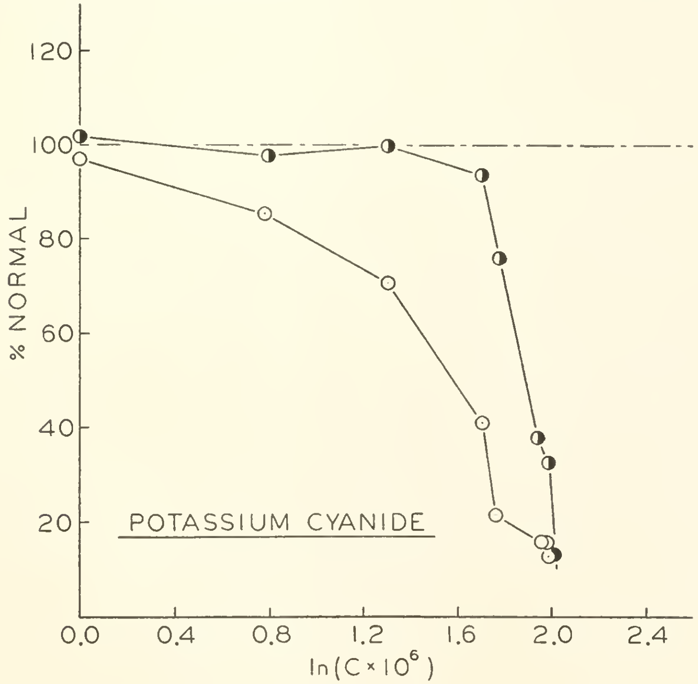

FIGURE 3. The effect of cyanide on rate ($1/t$) of regeneration (open circles), and on length of the regenerating primordium (half-closed circles). Data from experiment 8, Table II.

data on regeneration rates at various oxygen tensions. Table III gives the calculations made from Barth's experiment 7 on young stems comparable to the material used in the present study. There are not enough data on the effect of low oxygen tensions to determine definitely whether unavailability of oxygen acts in the same way as cyanide. The indication is however that cyanide involves other factors, since at 2.4 cc. of oxygen per liter a 22 per cent decrease in length is accompanied by only a 28 per cent increase in time to constriction. The interesting fact to emerge from Table III, in any case, is that at high oxygen tensions the increased regeneration rate, which can go as high as 148 per cent of normal, results in major part from increases in the mass of tissue transformed. Thus at 14.3 cc./liter the L factor is 34 per cent above normal, whereas the $1/t$ factor is increased only six per cent. This is in sharp contrast to the effect of cyanide, which over a wide range influences the regeneration rate by changing the $1/t$ factor while leaving the L factor relatively unaffected.

TABLE IV

The influence of temperature on regeneration of Tubularia

Temperature ° C.	Number of stems	Time (hours)	Rate		Length		Length/time	
			$1/t$	% of control	Micra	% of control	L/t	% of control
14. 20.5......	20	30.8	0.0325	114.0	1100	98.5	35.8	111.9
*18.7**......	*25*	*35.1*	*0.0285*	*100.0*	*1120*	*100.0*	*32.0*	*100.0*
13.5......	21	47.7	0.0210	73.8	1280	114.0	26.9	84.0
10.8......	19	57.2	0.0175	61.4	1400	125.0	24.5	76.7
7.0......	19	95.2	0.0105	36.8	1460	130.0	15.3	48.8
15. 20.5......	21	39.4	0.0250	109.0	1020	99.0	25.3	106.0
18.7......	*24*	*43.4*	*0.0230*	*100.0*	*1031*	*100.0*	*23.8*	*100.0*
13.5......	23	50.0	0.0200	86.9	1180	115.0	23.6	99.1
10.8......	17	64.5	0.0155	67.4	1290	125.0	20.0	84.0
7.0......	21	83.4	0.0120	42.2	1380	134.0	16.6	69.6
16. 20.5......	22	40.0	0.0250	119.0	1010	86.2	25.5	103.4
18.7......	*23*	*47.6*	*0.0210*	*100.0*	*1174*	*100.0*	*24.6*	*100.0*
13.5......	24	57.2	0.0175	83.5	1210	103.0	21.2	86.4
10.8......	18	66.6	0.0150	71.5	1260	107.2	18.9	77.8
7.0......	18	100.0	0.0100	47.6	1320	112.3	13.2	53.6
17. 20.5......	21	37.8	0.0265	111.0	1010	93.5	26.8	115.5
18.7......	*25*	*45.6*	*0.0238*	*100.0*	*1082*	*100.0*	*23.2*	*100.0*
16.0......	26	48.8	0.0205	86.1	1120	103.3	23.0	99.0
13.5......	20	55.5	0.0180	75.6	1180	109.0	21.2	91.5
10.8......	15	71.5	0.0140	58.9	1380	127.0	19.3	83.3
7.0......	17	100.0	0.0100	42.0	1410	130.2	14.1	60.8

* 18.7° was chosen as the control temperature since it is closest to the natural optimum of the material.

C. Temperature

The most striking exhibition of the independence of the length and time factors emerges from the data on the influence of temperature on regeneration,

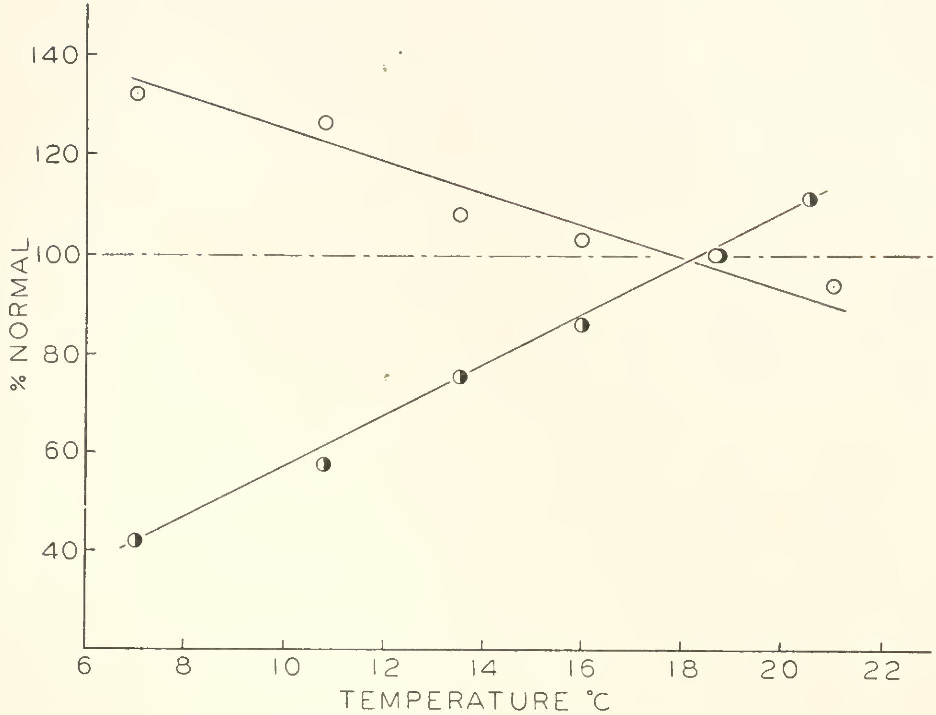

FIGURE 4. The effect of temperature on rate ($1/t$) of regeneration (half-closed circles), and on length of the regenerating primordium (open circles). Data from experiment 17, Table IV.

as summarized in Table IV; the actually opposite effects of temperature on the two rate components are illustrated in figure 4, which is a graph of experiment 17. It is of interest for later discussion to note that, in addition to moving in different directions, the two factors are independently sensitive to temperature changes. Table V shows the Q_{10} values calculated from 7° to 22° C. in five degree intervals. Each value in the "average" column was obtained from the results of four separate determinations. The average value over the entire range is also noted. The high values in each set of four experiments are included in the table to give an estimate of the upper limit of sensitivity for the two factors.

TABLE V

Temperature coefficients (Q_{10}) for length and rate in the regeneration of Tubularia

Temperature range ° C.	Length		Rate ($1/t$)	
	Average	High	Average	High
7–12	1.20	1.28	2.14	2.63
12.1–17	1.33	1.37	1.82	1.97
17.1–22	1.16	1.25	1.57	1.76
Average	1.23	1.30	1.88	2.12

It is evident that the Q_{10} values for $1/t$ are consistently higher both in the average and in the highest limits attained than those for L. It will also be noted that Q_{10} for length scarcely changes from low to high temperatures, while on the other hand the coefficient of the $1/t$ factor drops 0.57 from the lowest five degree interval to the highest.

Attempts have been made to explain such differences by invoking two separate processes unlike in their temperature sensitivities, one controlling the mass of tissue transformed and the other the time to constriction. Yet fundamentally length and time may be merely measurements of separate aspects of the same process. From this point of view it is more likely that the true explanation of the different responses to temperature is to be found in the purely numerical character of the terms which determine the two magnitudes, and in the way these magnitudes depend on parameters which vary with temperature. We hope to show in the discussion that this view is quite plausible. For the moment, however, it is sufficient to point out that the independent responses to temperature of L and t serve to emphasize further the independence displayed by the temperature coefficients.

DISCUSSION

That the mass of tissue involved in Tubularia reconstitution is independent of the time to constriction of the new primordium has been demonstrated with a variety of effective agents. It is evident then that L/t cannot be used as a measure of rate under different conditions unless it is accompanied by separate analyses of the behavior of the two components of the ratio. Thus for example the L/t ratio might be found constant over a range of temperature because of inversely proportionate effects on length and $1/t$. It is equally evident that the solution of the problem will not be reached by ignoring one or the other of the factors; for example, $1/t$ would not constitute an adequate description of the effect of high oxygen tension on the regenerative process.

From another point of view L/t, despite its correct dimensionality, may be expected to prove to be a relatively inaccurate measure of transformation rate. For the t in the definition is unlike similar factors in ordinary rate formulae, but is unique in the sense that it is determined by a stage in the development of the system. Its use implies the possibility of measuring rate by taking only two points (zero time and the time to the stage chosen) on the transformation-time curve and using the slope of the line connecting the two points as the rate of the process. Accuracy under such circumstances would be obtained only if the transformation-time curve were perfectly linear, i.e., if the rate up to the stage chosen were constant. In the case of a non-linear curve the approximation would become more and more crude with increasing deviation from linearity as well as with increasing distance between the selected points.

Thus the approximate nature of L/t as a rate measure may be easily recognized; the reasons for the relative independence of the length and time components, however, are not so evident. An insight into some of the factors involved may perhaps be gained by examining an attempt to measure the course of a simple chemical reaction by the same method, i.e., selecting only two points for observation, one at $t = 0$ and the other close to the end of the process, when the system becomes time-independent. So let us assume the following transformation

$$S \rightleftharpoons H, \tag{1}$$

the forward and backward reactions having velocity constants of k and k' respectively. Let s and h represent the initial concentrations of S and H, and x the number of moles of S transformed into H in t minutes. Then at the end of t minutes, $(s - x)$ is the concentration of S and $(h + x)$ is the concentration of H. After suitable rearrangements the transformation rate at any moment is given by

$$\frac{dx}{dt} = (k_1 - k'h) - (k + k')x. \tag{2}$$

Equation (2) may be integrated to yield the complete time course of the transformation, which takes the form

$$x = A - Ae^{-(k+k')t}, \tag{3}$$

where

$$A = \frac{k_1 s - k'h}{k + k'}. \tag{4}$$

It is evident from equation (3) that as t increases the exponential term becomes smaller and x approaches A, which represents its equilibrium value. Generally in measuring regeneration rates, and particularly in hydranth reconstitution, the stage chosen is one close to the end of the process, beyond which no further significant transformation occurs. A comparable t, in the simplified system being examined, would be one sufficiently large to make the exponential term numerically negligible. Let such a particular t be represented by T. At such time the amount of H present, a measure which would be comparable to the L in the regeneration rate formula, would be given by $(h + A)$. The "rate" analagous to L/t then takes the form

$$\text{rate} = \frac{h + A}{T}. \tag{5}$$

Examination of equations (3) and (4) reveals why the components of a rate so determined may be independent. The magnitude of t which will reduce the value of $Ae^{-(k+k')t}$ sufficiently to make x time-independent obviously depends on the magnitudes of A and $(k + k')$. Any experimental procedure which either increases or decreases k and k' proportionately will leave A, and consequently $(h + A)$, undisturbed, but will change the t (i.e., T) necessary to reduce the second term to insignificance. Under such conditions, the rate as defined by equation (5) would vary solely because of a changing denominator, the mass factor in the numerator remaining constant. On the other hand, an experimental procedure that varied s, the initial concentration of S, might from an observational point of view affect only the mass, since A is a function of s. Strictly speaking, any change in A also influences the T value, since A is included as a factor in the time-determining term. However, in any combination of an ordinary algebraic and an exponential factor, the latter quickly predominates in determining the numerical value of the product. Thus, unless the variation in s produced a very marked change in A, the T values before and after the change might not be experimentally distinguishable, even though the difference in the A values were easily detected.

It is hardly conceivable that the complex of processes leading to the reconstitution of a hydranth can have much resemblance to the simple reaction represented by equation (1). About the only properties the two have in common are that they both involve molecular transformation and that they both take time to arrive at a time-independent state. The fact that the "rate" of the chemical reaction as defined by equation (5) exhibits many of the characteristics experimentally found for L/t is most likely inherent in the basic similarities underlying the two definitions. The approximate nature of both rate formulations would tend to conceal any differences in the processes they are used to measure. In any case, the above analysis strongly suggests the possibility that some of the peculiarities of the L/t rate found experimentally with the various reagents may in part be characteristic of the definition rather than of the mechanism of regeneration.

It is worthy of note that the decrease in L observed at higher temperatures is not shown by the mass factor of the rate described by equation (5). The numerical reason for the constancy of the numerator with temperature variation is that a temperature change can only yield proportionate changes in the forward and backward velocity constants; since these appear to the same powers in both the numerator and the denominator of A, the net result is that A remains constant. Underlying this behavior is the fundamentally important fact that equation (1) represents a reaction occurring in a closed system and as such its equilibrium point, as far as the concentration of reactants is concerned, is independent of temperature.

If the temperature variation of L is to be examined, therefore, it is necessary to study an open system whose time-independence is maintained by a constant flow of material or energy through it. As has already been pointed out by Burton (1939), open systems are far more likely than closed systems to possess kinetic characteristics typical of living organisms, simply because the latter are themselves open systems, and approach steady states rather than true thermodynamic equilibria. If instead of equation (1) we introduce a source O for S, and a sink P for H, the system becomes an open one, since the concentrations of S and H now become dependent on parameters external to the transformation, namely the levels of O and P. For purposes of simplicity we shall assume that the back reaction k' is either zero or at least negligibly small as compared with the forward reaction. This is plausible whether we consider the S to H transformation itself the energy-yielding reaction which leads to hydranth synthesis, or whether we regard the transformation as being driven by some other energy-yielding reaction. In the first case the transformation would tend to be relatively irreversible, in the second the coupled energy-yielding reaction would tend to make the reverse reaction from H to S relatively insignificant. Instead of (1) then we may write

$$O \overset{k_o}{\leftrightarrow} S \overset{k}{\rightarrow} H \overset{k_p}{\leftrightarrow} P. \tag{6}$$

The velocity constants, k_o and k_p, connecting O to S and H to P respectively, are taken to represent both the forward and the backward velocities of the two reactions they govern. This assumption of equal forward and backward velocities, while not necessary for the analysis, avoids the undue complication that would result from too many constants. In addition, if O represents a type of source in which S-substrate diffuses from O to the site of the reaction, and P represents a type of sink toward which the produced H diffuses, then the assumption of equality would exactly describe the situation. Letting C_o, C_s, C_h, and C_p represent the concentration levels of O, S, H, and P, the following equation may be written for the kinetics of the transformation of S into H

$$\frac{dC_s}{dt} = k_oC_o - C_s(k_o + k). \tag{7}$$

Integrating equation (7) yields the time variation of the concentration of S, which is given by

$$C_s = B + Ge^{-(k_o+k)t}, \tag{8}$$

where

$$B = \frac{k_oC_o}{k_o + k} \tag{9}$$

and

$$G = (C_{s_o} - B), \tag{10}$$

C_{s_o} being the concentration of S at zero time. Equation (8) is formally identical with equation (3). It is readily seen from equation (8) that B represents the time-independent or steady state value of C_s and is analagous to A of the previous case. Setting up for the present system a rate similar to L/t and equation (5), we may write

$$\text{Rate} = \frac{B}{T} \tag{11}$$

in which T has the same significance as in (5).

Thus again, by the same arguments used in the analysis of a reaction going to equilibrium, which need not be repeated here, it becomes evident that differential effects on either mass or T factor in (11) may be obtained. Thus a suitable experimental procedure which affected only C_o would manifest itself by marked changes in B and weak changes in T. Such may well be the explanation for the results of Barth's experiments on the effects of high oxygen tension, in which the length was increased strongly and the T factor very little. The implication that high oxygen tension raises the level of the source for S (i.e., C_o) fits into the hypothesis proposed by Barth (1940) that oxygen is directly involved in the synthesis of a substance S whose transformation yields hydranth.

Again as in the previous case, treatments which affect the velocity constants k_o and k would result in marked variations in T as compared with B. On the basis of this analysis, one would then interpret the results with ethyl urethane, sodium cyanide, and sodium azide in terms of decrease of the values of velocity constants by poisoning of the enzymes involved in the transformation. Phenyl urethane and chloretone on the other hand, in addition to decreasing the velocity constants, also appear to lower the C_o value by interfering with the synthesis of the substance S or its immediate precursors.

Since increases in temperature raise the values of velocity constants, it is evident why lower T values are found at higher temperatures. A possible reason for the decrease of mass transformed with increasing temperatures may be found in an examination of the effects of variations in k_o and k_1 on B, the steady state value of C_s. The change in B for variations in the velocity constants is given by

$$dB = \frac{C_s(kdk_o - k_odk)}{(k_o + k_1)^2}. \tag{12}$$

We are concerned here with temperature increases; consequently both dk_o and dk will be increments, i.e., positive quantities. All other factors of the right-hand member of (12) being positive, it is clear that dB will be either positive or negative according as $(kdk_o - k_odk)$ is either positive or negative. Thus the mass factor will decrease with increasing temperature if

$$kdk_o - k_odk < 0. \tag{13}$$

Inequality (13) can be satisfied in several ways. Thus for example, if k_o were of the nature of a diffusion coefficient and k the velocity of a chemical reaction, then for a given rise in temperature the increment in k_o would be about half that realized by k. Inequality (13) then becomes

$$dk\left(\frac{k}{2} - k_o\right) < 0$$

and is satisfied if $k < 2k_o$. If on the other hand the two processes have the same temperature coefficients, so that dk were equal to dk_o, inequality (13) could be satisfied if k_o were greater than k. Whatever the intimate details of the situation may actually be, it is evident that opposing responses of mass and time found in regeneration may plausibly be explained as an expression of the operation of an open system approaching a steady state. Decreases of size with increasing temperature are not confined to regeneration in Tubularia, but are a general phenomenon of development and have been studied in other forms including the trout (Gray, 1928; Merriman, 1935), the whitefish (Price, 1940), and the frog (Chambers, 1908).

The different sensitivities to temperature of length and time apparent in the Q_{10} values given in Table V find their most plausible explanation in the algebraic composition of the two terms that determine them. We have already noted that in the special case in which the Q_{10} values of k_o and k are equal, the Q_{10} for the mass would be unity since proportional increases in these constants would cancel out and leave B unchanged for any temperature rise. On the other hand T, since it is determined by the sum of k_o and k, would be affected more or less strongly according to the magnitude of the temperature change. This same difference in response will be carried over to the more general case where the temperature coefficients of k_o and k differ. By the very nature of the dependence of B on these constants, increases or decreases in the constants cannot result in as marked changes in B as they would in T, which is exponentially dependent on their sum.

It is apparent from this discussion that neither $1/t$ nor L/t can be treated as ordinary rates. However the L/t definition of regeneration rate, if supplemented by a further analysis of the separate behavior of L and $1/t$, can yield interpretable information on the regeneration process. The omission of the mass factor is surely not justifiable on the grounds of "correcting" the L/t definition. The latter will in many cases yield information which the "corrected" rate would miss entirely.

SUMMARY

1. Data are presented which show that various agents produce differential effects on the length of the regenerating primordium of a Tubularia hydranth and on the time to the constriction of the primordium from the rest of the stem.

2. The significance of this independence of length and time for the L/t formulation of regeneration rate is discussed.

3. The differential effects are interpreted in terms of a reaction approaching a steady state in an open system.

4. The criticisms of the L/t definition and the proposed substitution of $1/t$ are discussed in terms of the above analysis. It is concluded that the L/t definition, if supplemented by a further analysis of the independent behavior of L and $1/t$, provides a useful and informative measurement of regeneration.

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NEUROSECRETION

VI. A COMPARISON BETWEEN THE INTERCEREBRALIS–CARDIACUM–ALLATUM SYSTEM OF THE INSECTS AND THE HYPOTHALAMO–HYPOPHYSEAL SYSTEM OF THE VERTEBRATES[1]

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A comparison of data on the secretory activity of nerve cells in invertebrates with those obtained from corresponding studies in vertebrates revealed an interesting parallelism between the intercerebralis-cardiacum-allatum system of insects and the hypothalamo-hypophyseal system of vertebrates. The functional mechanism involved cannot be fully explained at present; but the observations are in themselves intriguing and offer a point of departure for the discussion of certain neuro-endocrine relationships.

INSECTS

In the larvae of muscoid Diptera the ring-gland, an endocrine organ concerned with development (Hadorn, 1937; Hadorn and Neel, 1938; Burtt, 1938; Becker and Plagge, 1939; Vogt, 1942a; Gloor, 1943; Bodenstein, 1943a, 1943b, 1944), contains the elements of two glands, the corpus cardiacum and the corpus allatum (Scharrer and Hadorn, 1938; Vogt, 1942b; Day, 1943; Poulson, 1944). In other insects these two components form more or less individual organs. In *Leucophaea maderae,* a species used in the present study, the corpora cardiaca and allata are paired organs which, as in other representatives of the Orthoptera (De Lerma, 1937; Hanström, 1940), lie dorsal to the esophagus behind the brain. The anterior portions of the elongate corpora cardiaca form part of the wall of the dorsal blood vessel. The posterior ends of the corpora cardiaca are in contact with the corpora allata, which lie more laterally than the former. In Leucophaea the two glands are not, as in other species, separated by a nervus corporis allati but constitute an almost continuous mass of glandular tissue.

Histologically the corpus cardiacum can be easily differentiated from the corpus allatum. Cardiacum tissue contains nervous as well as glandular elements, whereas there is no indication of a nervous component in the corpus allatum. In Leucophaea the corpus cardiacum is to a varying degree filled with deeply staining colloid masses; in older specimens the gland may be replete with such acidophil substances. By comparison little material that can be interpreted as a secretory product is, as a rule, found in the corpus allatum. The physiological significance of the variations of the histological appearance of both the corpora cardiaca and corpora allata is not clear at present; but it is evident that they are both glands.

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[1] This research was aided by a grant made to Western Reserve University by the Rockefeller Foundation.

The corpora cardiaca receive a well defined fiber bundle (nervus corporis cardiaci, Pflugfelder, 1937; nervus corporis cardiaci I, Hanström, 1940; nervus occipitalis, Nesbitt, 1941) from the pars intercerebralis of the protocerebrum. In Leucophaea the fibers turn from their origin antero-medially and downward. Most of them, perhaps all, cross in the midline and continue toward the base of the brain. Thence the fiber bundle turns backward and shortly after leaving the brain enters the corpus cardiacum. The bundle can be followed all the way through the gland, which it innervates (Fig. 1). It seems that some of the fiber components enter the corpus allatum of the same side where they are distributed.

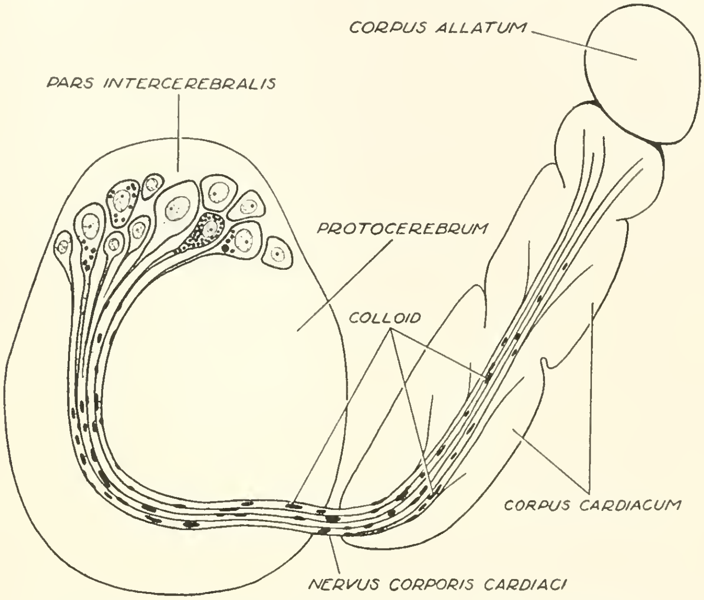

Figure 1. Diagram of the intercerebralis-cardiacum-allatum system of an insect.

The pars intercerebralis of the insect brain is distinguished by the occurrence of secreting nerve cells. Such cells have been found in Hymenoptera (Weyer, 1935; Scharrer, 1937), Hemiptera (Hanström, 1938; Wigglesworth, 1940), Lepidoptera (Day, 1940a), Coleoptera, Neuroptera, Trichoptera, and Diptera (Day, 1940b; Vogt, 1942a).[2] In the Orthopteran *Leucophaea maderae,* for instance, the medium sized nerve cells of the pars intercerebralis contain distinctly staining inclusions varying in size and number. There may be only two to three granules present in one cell, or they may be so numerous that they fill the entire cell body. Not only the number of granules in different cells, but also the number of secreting elements varies in different specimens. There may be numerous cells on either side of the midsagittal plane containing granules or there may be only a few such cells. The secretory material is in some cases concentrated near the axon hillock, and may continue for a certain distance into the axis cylinder, which in this case appears wider than in nerve cells of comparable size without secretory granules.

[2] R. P. Holdsworth found neurosecretory cells in the pars intercerebralis of Pteronarcys, a representative of the Plecoptera (personal communication).

The colloid granules in the pars intercerebralis resemble in size and stainability those found and previously described in the neurosecretory cells of the subesophageal ganglion (Scharrer, 1941a). The neuroglandular cells are larger in the subesophageal ganglion than in the pars intercerebralis.

Along the fibers of the nervus corporis cardiaci colloid masses are found in varying, sometimes very great amount, particularly in older specimens of Leucophaea. The colloid content of the fiber bundle permits its tracing and differentiation from other tracts (Fig. 1).

The concept of an anatomical system formed by the pars intercerebralis and the corpora cardiaca and allata facilitates the understanding of the hormonal regulation of postembryonic insect development. Several hormones derived from different sources have been demonstrated to control growth and differentiation in various groups of insects. The corpus allatum is known to furnish an "inhibitory hormone" in Hemiptera (Wigglesworth, 1934, 1936, 1940), Orthoptera (Pflugfelder, 1937; Pfeiffer, 1942; Scharrer, 1944), Lepidoptera (Bounhiol, 1939; Piepho, 1943), and Coleoptera (Radtke, quoted from Piepho, 1943). A substance originating in the brain brings about molting in Hemiptera (Wigglesworth, 1940), and pupation in Lepidoptera (Kopeć, 1922; Caspari and Plagge, 1935; Kühn and Piepho, 1936) and Hymenoptera (Schmieder, 1942). Additional centers in the thorax (or possibly upper abdomen) have been claimed to play a role in pupation and imaginal differentiation of Lepidoptera (Hachlow, 1932; Bounhiol, 1938; Bodenstein, 1938; Fukuda, 1940; Piepho, 1943) and Neuroptera (Ochsé, 1944). In the highly specialized muscoid Diptera the ring-gland containing both corpus cardiacum and allatum controls growth, molting (Bodenstein, 1944), pupation (Hadorn, 1937; Hadorn and Neel, 1938; Becker and Plagge, 1939; Vogt, 1942a), and imaginal differentiation (Bodenstein, 1943b), whereas the brain is said to have no influence on these processes. There is indirect evidence that at least the substance causing puparium formation is produced by the cardiacum component of the ring-gland (see Scharrer, 1941b). Finally, removal of the corpora cardiaca in Orthoptera causes a retardation of molting (Pfeiffer, 1939).

In an attempt to reconcile some of the seemingly divergent data it may be useful to discuss first the various hormones named, and second their source in the organism.

Concerning the hormones controlling postembryonic insect development two interpretations are possible: (*a*) Each developmental step is brought about by one or several specific hormones. Accordingly there would exist molting, pupation, and metamorphosis hormones. (*b*) There are two types of hormones interacting during development. The one type activates the imaginal potencies in a measure regulated by the responsiveness of the developing tissue and thus brings about periodic growth and differentiation. Factors of this type are called in this paper "growth and differentiation hormones." The other type, juvenile or inhibitory hormone, activates the "juvenile," i.e. larval potencies of the cells, and in this way prevents the onset of metamorphosis. According to this concept, first formulated by Wigglesworth (1934, 1936, 1940), the presence of both factors in adequate proportion causes larval (nymphal) molting, whereas in the absence of the juvenile factor metamorphosis takes place. There is strong evidence that this "dualistic" mode of regulation exists not only in hemimetabolous (Hemiptera, Wigglesworth, 1934, 1940; Orthoptera, Pflugfelder, 1937, 1940; Pfeiffer, 1942; Scharrer, 1944), but also in holometabolous forms (Lepidoptera, Bounhiol, 1939; Coleoptera, Radtke, quoted from Piepho, 1943).

In holometabolous insects, then, the effect ascribed by certain authors to a "molting hormone" would actually result from the combined action of two hormonal factors, a juvenile hormone and a growth and differentiation hormone. Pupation and metamorphosis would take place in the presence of one or more differentiation factors alone.

As sources of the hormones controlling insect development three organs in the head region of insects are known at present: (1) the glandular corpora allata, (2) the corpora cardiaca, consisting of nervous and glandular elements, (3) the pars intercerebralis of the brain containing glandlike nerve cells.

In contrast to the known action of the corpus allatum which is the source of the juvenile hormone (see p. 244), the role of the two remaining centers has been less well understood. In one group of insects the brain seems to produce a hormone (or hormones) which in another group is provided by the corpus cardiacum. These two sources do not need to be treated as two separate centers of glandular activity, different as they may seem at first sight. On the basis of their unusual morphological relationship it is proposed to consider them as components of one neuro-endocrine complex whose role in the developing insect is the regulation of growth and differentiation.

As to the mechanism of this glandular complex there are two possibilities. Either both the brain and the corpus cardiacum cooperate in the elaboration of growth and differentiation factors, or in different animals the one or the other component has become the predominant hormone source. Considering the variability in the development of neuroglandular organs in the insect head one may expect to find examples for either alternative among the various groups of insects.

The first possibility has to be considered, if extirpation of one of the two glandular centers leads to disturbances but not to a complete interruption of the endocrine mechanism. For instance, it is known that cardiacectomy in Melanoplus (Orthoptera, Pfeiffer, 1939) delays but does not entirely prevent molting.

There are data that indicate the second possibility, i.e. an autonomous action of either the brain or the corpus cardiacum. In nymphs of Rhodnius (Hemiptera, Wigglesworth, 1940) brain implants cause molting in the absence of the corpus cardiacum. In Drosophila and Calliphora the ring-gland (in all probability its cardiacum component) furnishes growth and differentiation hormones, whereas the brain alone has little or no effect. Most of these data in Diptera (Hadorn, 1937; Hadorn and Neel, 1938; Burtt, 1938; Becker and Plagge, 1939; Vogt, 1942a; Gloor, 1943; Bodenstein, 1943a, 1943b, 1944), as well as observations made in other groups of insects, do not preclude, although they do not prove a collaboration between pars intercerebralis and corpus cardiacum in the production of growth and differentiation hormones.[3]

All these data and considerations concern the developing insect. In the adult the corpora allata control egg development (Wigglesworth, 1936; Pfeiffer, 1939; Thomsen, 1940; Vogt, 1940; Scharrer, 1943) and color change (Pflugfelder, 1939);

[3] Further information will be necessary about the identity and mode of action of certain thoracic centers, mentioned on p. 244, before they fit into the present concept of the hormonal control of insect development (see also Richards, 1937).

the functional significance of the intercerebralis-cardiacum complex is still unknown. However, the production of physiologically active substances by the intercerebralis-cardiacum complex also in the imago is indicated by the fact that extracts from either component in Periplaneta yield chromatophorotropic responses in crustaceans (Brown and Meglitsch, 1940).

In summary, the intercerebralis-cardiacum-allatum system furnishes two types of hormonal factors which by their interaction control the rate of insect development. The one type, i.e. growth and differentiation hormone (or hormones), originates in the neuroglandular intercerebralis-cardiacum complex, the other (juvenile, inhibitory hormone)in the corpus allatum. This seems to be the most satisfactory interpretation of the numerous existing observations. As has been indicated (p. 245) it is not the only one possible.

VERTEBRATES

The pituitary gland consists of two components, the pars buccalis (anterior lobe), and the pars nervosa (posterior lobe). The latter receives its innervation from nuclei in the hypothalamus; how many of the nerve fibers also end in the anterior lobe, is not definitely known, and it seems to vary in different groups of vertebrates. In the fishes, the nucleus preopticus and the nucleus lateralis tuberis send their fibers to the hypophysis. The nucleus preopticus alone innervates the gland in the amphibians. In the reptiles the nucleus preopticus is divided into two nuclei, the nuclei supraopticus and paraventricularis (Meyer, 1935). The cells of these two nuclei send their axons to the pituitary gland in the reptiles and the mammals. The significance of these hypothalamic nuclei as nervous centers controlling pituitary activity has been studied carefully in some species such as the cat, particularly by Ranson and his collaborators (Ranson and Magoun, 1939).

The same cells which through their axons innervate part or all of the pituitary gland have been shown in a number of vertebrates to exhibit characteristics of gland cells (Scharrer and Scharrer, 1940). This means that the cells pass through cycles of secretory activity during which they produce granules and colloid droplets.

The question arises whether this secretory activity of the nerve cells is connected with the control of the pituitary gland or has no relation to this function. The latter would be difficult to understand. The nervous control of the neural lobe is known to be of great importance for the normal function of this organ. It seems inconceivable that the cells which innervate the gland could themselves change into gland cells to the extent observed in some animals if the secretory activity of these nerve cells would serve a purpose unrelated to the activity of the pituitary gland. Such an independent glandular function would probably interfere with the task of the cells of innervating the hypophysis.

The alternative, that the secretory activity of the neurons is part of the mechanism through which the hypothalamic nuclei exert control over the pituitary gland, would appear to be more acceptable. Evidence to support this view may be seen in two kinds of observations: (1) The secreted material can be traced along the axons to the hypophysis. (2) There is a seasonal cycle in certain cases of neurosecretory cells which may have a significance with regard to seasonal cycles in hypophyseal activity.

Granules and droplets, discharged by secreting nerve cells, have been traced along the axons in a number of species (Fig. 2). In the catfishes, *Noturus flavus* and *Amciurus nebulosus*, the nucleus preopticus and the nucleus lateralis tuberis both consist of secreting nerve cells (Palay, 1943). Acidophil granules and droplets of the same kind as produced by these cells are found all along the fibers from the preoptic nucleus to the pituitary gland. This tract can actually be differentiated from other fiber connections by the granules which in Masson preparations stain red and mark the bundle as clearly as the black granules would in a successful Marchi preparation (Palay, unpublished).

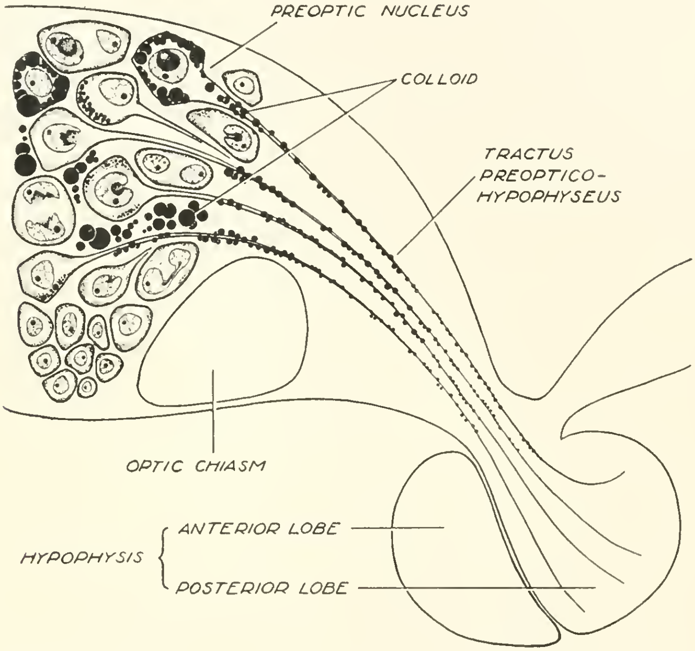

FIGURE 2. Diagram of the hypothalamo-hypophyseal system of a vertebrate.

The granules along the axons of the preoptic nucleus can be seen also in other fishes, such as Tinca (Scharrer, 1936), Fundulus, and Centropristes. Likewise in amphibians, for instance in the toad, the granules are attached to the axons in bead-like arrangement for a long distance from the cell of origin. In reptiles, particularly in snakes, the fiber tract between the supraoptic nucleus and the hypophysis may be filled with colloid droplets as in the catfish. Also in the human supraoptic nucleus cells have been seen with acidophil granules along the axon some distance from the cell body (Gaupp and Scharrer, 1935).

In the vertebrates a considerable amount of experimental work has been done, but the physiological role of the material secreted by the nerve cells is still unknown. All that can be said at present is that if the acidophil material discharged by the nerve cells contains an active principle, it appears to be directed toward the hypophysis.

It should be mentioned here that a number of investigators have suggested a migration in the opposite direction, i.e. of hypophyseal colloid from the pituitary gland to the hypothalamus (Edinger, 1911; Cushing, 1925; Collin, 1928; Popják, 1940). There is no doubt that this actually takes place. The hypophyseal colloid can be differentiated from the colloid of the nerve cells in that it stains slightly different and appears in the shape of irregular masses instead of sharply defined granules. This hypophyseal colloid cannot be traced very far, and it is questionable whether it reaches the hypothalamus; but the possibility that colloid may be exchanged in both directions must be acknowledged. The significance of such an exchange is largely obscure, but a close interrelation between the activity of the hypophysis and that of neurosecretory cells in the hypothalamus is suggested.

A seasonal cycle of the secretory activity of neurosecretory cells has been found so far only in one species of teleosts. The cells of the nucleus lateralis tuberis of the tench (*Tinca vulgaris*), a close relative of the carp, show no secretory activity during the winter months. It is very conspicuous during the summer months with gradual increase in spring and decrease in fall (Scharrer, 1936). In catfishes (*Amciurus nebulosus* and *Noturus flavus*), collected during the past three years, no corresponding cycle was found (Scharrer and Palay, unpublished). The pituitary gland of fishes is also subject to seasonal changes (Bock, 1928; Matthews, 1939; Evans, 1940). Whether and in which way the cyclic hypophyseal phenomena are related to those taking place in the nucleus lateralis tuberis is not known at present. The data available require closer investigation.

Consequently it is proposed to follow the suggestion of physiologists and pathologists who have been considering the hypothalamic nuclei of higher animals together with the neural lobe as an interdependent system. Such a hypothalamo-hypophyseal system could be assumed to have originated from a hypothetical situation in which from one neuroglandular area in the brain the secreting hypothalamic nuclei and the pars nervosa of the hypophysis have been derived. The exchange of colloid, whatever its functional meaning may be, could be considered as a remnant of the original connection. Charlton (1932) has presented evidence that in phylogeny the nucleus preopticus of the fishes has migrated rostrally. The cauda of the nucleus preopticus in fact points toward the hypophysis, and the irregularly occurring nucleus lateralis tuberis is still in very close proximity to the pituitary gland.

Discussion

A comparison of the hypothalamo-hypophyseal system of vertebrates with the intercerebralis-cardiacum-allatum system of insects reveals a parallelism which is the more striking because insect and vertebrate organs differ so greatly that no true organ homology can exist between these phyla.

The hypothalamic nuclei of the vertebrates have their equivalent in the pars intercerebralis of the insects. In both centers neurosecretory cells are found, and both send nerve fibers to innervate complex endocrine organs, i.e. the pituitary gland and the corpora cardiaca and allata. In both the vertebrates and the invertebrates these nerve fibers contain colloid which can be traced from the nerve cells all the way to the glands innervated by them.

The endocrine glands too are comparable as Hanström (1941) has pointed out. The hypophysis produces a number of well-known hormones influencing growth, gonadal development, chromatophores, etc. The corpora cardiaca and allata control processes of equivalent importance in the life of insects, such as growth and metamorphosis, egg development, color change, etc. Evidently the corpora cardiaca and allata play a role in insects similar to that of the pituitary gland in vertebrates.

Both glands are composite structures. The pituitary consists of a neural portion which forms the posterior lobe, and a glandular portion which forms the anterior lobe. These two components become associated to a varying extent; they are most closely connected in the teleosts where the pars nervosa penetrates the pars glandularis. In the insects the corpora cardiaca are comparable to the neural portion, the corpora allata to the glandular portion of the hypophysis. The corpora cardiaca and allata become associated in most insects; in the muscoid Diptera they form an organ (ring-gland) in which the two components can be differentiated only histologically (Scharrer and Hadorn, 1938; Vogt, 1942b; Day, 1943; Poulson, 1944).

The parallelism in the organization of the two systems here compared could be merely a coincidence. However, it seems more likely that the comparison is significant in that it indicates a fundamentally similar relationship between the "master glands" and the central nervous system in invertebrates and vertebrates.

SUMMARY

The hypothalamo-hypophyseal system in vertebrates is in many respects similar to the intercerebralis-cardiacum-allatum system in insects.

(1) In vertebrates the hypothalamic nuclei innervating the pars nervosa of the pituitary gland contain secreting nerve cells. In a number of species colloid droplets can be traced along the axons from the neurosecretory cells of the hypothalamus to the hypophysis.

(2) In insects the pars intercerebralis of the protocerebrum contains neurosecretory cells. A bundle (nervus corporis cardiaci) innervating the corpus cardiacum and probably also the corpus allatum originates in the pars intercerebralis. In Leucophaea (Orthoptera) as in the vertebrates, colloid can be traced from the secreting nerve cells of the pars intercerebralis to the corpora cardiaca all along the nervus corporis cardiaci.

(3) On the basis of these morphological relationships the hypothalamic nuclei (nucleus preopticus and its homologues) and the pars nervosa of the hypophysis appear as one closely interconnected system. Likewise the pars intercerebralis and the corpus cardiacum of insects may be viewed as one neuro-endocrine complex rather than as two separate sources of hormones. In this way certain seemingly inconsistent data concerning the endocrine control of development in insects can be better understood (see p. 244).

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MECHANISM OF PIGMENT DISPLACEMENT IN UNICELLULAR CHROMATOPHORES

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Introduction

Several hypotheses have been proposed regarding the basic mechanism which determines the flow of pigment granules back and forth in the protoplasmic branches of unicellular chromatophores. The melanophores of vertebrates, for example, have been regarded as amoeboid cells with pseudopodia which extend and retract alternately (Hooker, 1914); and as modified visceral muscle cells (Spaeth, 1916) More recently, Shanes and Nigrelli (1941) have related the movement of the melanin granules to processes occurring in some birefringent material which appears to lie between and around the melanophores proper.

The present investigation deals with the action of hydrostatic pressure on the melanophores of the isolated scale of *Fundulus heteroclitus,* and the work forms a sequence with several previous studies. Amoeboid movement (Brown and Marsland, 1936), the cleavage movements of various marine eggs (Marsland, 1938 and 1939a), and protoplasmic streaming in plant cells (Marsland, 1939b) were all found to be activated by intracellular processes of gelation and solation (Marsland, 1942). Protoplasmic gelations have proved to be uniquely susceptible to the influence of hydrostatic pressure (Marsland and Brown, 1942), and consequently an opportunity is afforded for studying the role of such reactions in the behavior of the melanophore.

Experimental Results

Effects on pulsating melanophores

Pulsation of the melanophores of the isolated scale was induced by a modification[1] of the method of Spaeth (1916), and the scale was then placed in the microscope-pressure chamber (Marsland and Brown, 1936). This chamber permits the melanophores to be viewed at a magnification of 600 diameters at pressures ranging up to 8000 lbs./in.^{2}.

At 1000 lbs./in.^{2} the pressure effect upon the pulsating cells is scarcely discernible; but at 2000 lbs. the change becomes very obvious. The flow of the pigment granules back and forth in the branches of the melanophores continues, but the amplitude of each pulsation is plainly reduced. This reduction is confined, however, to the central ends of the branches of the melanophores. The outflow of pigment granules continues to reach the distal ends of all branches, but the inflow reverses itself before the granules have fully reached the central protoplasmic mass. Thus at the maximum of each concentration the melanophore displays a number of short stumplike excrescences which correspond to the number of branches which are fully developed at maximum expansion.

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[1] The scales were scraped from the dorso-lateral surface of the fish and placed in N/10 normal NaCl solution for 15 mins. before transfering to N/10 BaCl for seven minutes. After this the scales were returned to fresh NaCl solution in which the pulsations would start in about one hour and continue for more than two hours. A thorough two-fold washing of the scales in separate samples of each solution was done before they were introduced into the pressure chamber.

At pressures between 3000 and 6000 lbs./in.^{2}, the amplitude of the pulsations is reduced more and more, entirely at the central end. The outflow still reaches the tips of the branches, but the inflow, at the higher pressures, is so restricted that almost the full length of each branch remains when the concentration of pigment is complete. Finally, at pressures between 7000 and 8000 lbs./in.^{2}, the pulsations cease altogether. Now all the melanophores remain in their completely expanded form.

If the pressure is suddenly released, an immediate concentration phase always sets in. This "release contraction" is very rapid, and it endures somewhat longer than the "contraction" which occurs in the ordinary rhythm of an uncompressed specimen.

Effects on contracted[2] *melanophores*

Since pressure inhibits and finally abolishes the contraction phase of the pulsation cycle, it was of interest to determine how the melanophores might react in the presence of reagents which induce the pigment to remain in the concentrated state. Most of these experiments were done with scales immersed in N/10 KCl solutions, although the same results were also obtained with adrenalin (1 : 1000). The melanophores of uncompressed control scales in these solutions remained in a fully contracted state for a period far in excess of the time required for the experiments.

The results of these experiments are indicated in figure 1. The several photographs are of the same melanophore successively exposed to different degrees of pressure. The response to increased pressure is always an immediate expansion, and the degree of this expansion bears a direct relation to the intensity of the pressure up to 7000–8000 lbs./in.^{2}. At this level a maximum dispersal of the pigment is always observed.

The degree of expansion depends directly upon the intensity of the pressure. The same configuration is reached at a certain pressure regardless of whether the melanophore expands to this point, as a result of increasing the pressure, or contracts as a result of decompressing. Also when a fixed pressure is maintained, the characteristic degree of expansion is maintained for many minutes. But whenever the pressure is completely released, the melanophores return immediately to a fully contracted state.

Experiments with denervated melanophores

Since Parker (1934) has shown that the terminal branches of the nerves which supply the melanophores retain some influence on the contractile state even after all connections with the central nervous system have been severed, it was necessary to determine whether or not the action of pressure is mediated through the activity of the surviving nerve remnants. Therefore the foregoing experiments were repeated, using melanophores in which complete denervation was assured.

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[2] The term *contraction* will be used to designate the process whereby the pigment undergoes concentration in the melanophore, despite the fact that this process is not entirely comparable to the contraction of a muscle. This usage seems justifiable in view of the fact that pigment concentration probably does involve a contraction of the plasmagel system of the chromatophore, although the cell as a whole does not contract (see p. 259).

For this purpose the method of Parker was used. Broad dark bands were established in the caudal fins of ten medium sized Fundulus, by making dorso-ventral cuts completely through the fins about two mm. caudal to the origin. After ten days the bands were not quite as wide as originally, but they were still clearly discernible as darker areas in light-adapted fish and as lighter areas in dark-adapted specimens. In accordance with Parker's conclusion, these observations indicate that the degeneration of the residual nerves is complete within ten days, and that longer periods must elapse before secondary innervation can occur.

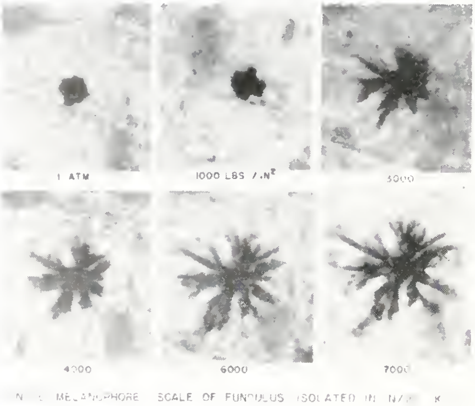

FIGURE 1. Progressive inhibition of contraction by hydrostatic pressure (courtesy of Iowa State College Press, Ames, Iowa).

On the eleventh day a small square section of the tail-fin, so selected that about half the area was derived from the denervated region, was excised, washed in three changes of N/10 KCl, and placed in the pressure chamber, which also contained the same solution. All the melanophores of the excised piece were fully contracted and there was no discernible difference between the melanophores of the innervated and denervated areas. This proves that the contracting action of the KCl solution does not depend upon the survival of the terminal nerves.

Different pressures, ranging up to 8000 lbs. in., gave exactly the same results as were described previously. Both groups of melanophores, regardless of the presence or absence of surviving nerves, gave the same degree of expansion with each increment of pressure, and in both groups a full expansion was reached at 7000–8000 lbs. This proves that the pressure acts directly upon the melanophores per se, rather than indirectly, via surviving nerve elements.

Centrifuging experiments

The foregoing experiments indicate that pressure exerts an inhibiting effect upon some protoplasmic reaction which determines the contractile state of the melanophore. Apparently the equilibrium of this reaction can be shifted by each increase or decrease of pressure in the range up to 8000 lbs./in.^{2}. In this respect the activity of melanophores closely resembles amoeboid movement, cleavage and cyclosis. And since these physiological activities are known to be determined by sol-gel changes occurring in the protoplasm, centrifuging experiments were undertaken, on the hypothesis that similar factors are involved in the present case.

It soon became apparent that melanophores are very unfavorable for measuring gelational changes in the protoplasm. Very high centrifugal forces must be employed before any sign of pigment displacement can be obtained and, due to the highly irregular form of the melanophore, quantitative measurements of the degree of displacement are quite impossible. However, certain qualitative indications were obtained when the isolated scales were subjected to a centrifugal force of 70,000 gravity in an air turbine ultracentrifuge.[3]

In the centrifuging experiments it was necessary to find a method for holding the centrifuged scale in a position such that the anterior-posterior axis of the scale (and consequently the plane occupied by each melanophore and its processes) was parallel to the centrifugal axis. This was accomplished by rolling the scale into the form of a cylinder and inserting it into a short length of pyrex capillary tubing, sealed at the centrifugal end. The diameter of the lumen of the tubing was about half the width of the scale. Consequently when the elastic scale begins to unroll, its outer surface becomes firmly pressed against the inner surface of the tubing. The external diameter of the pyrex tubule was only slightly less than the internal diameter of the metal jacket in the head of the ultracentrifuge, and consequently the tubule axis and the axis of the centrifuge were approximately identical. Both the metal jacket and the pyrex tubule were filled with the immersion solution, and this arrangement tended to reduce the force of the impingement of the glass tubule upon the bottom of the metal jacket.

Assuming that the resistance to the displacement of the melanin granules through the protoplasm of the melanophore provides an index of gelation, the experiments support the view that the protoplasm is set more firmly in the contracted than in the expanded melanophore. Using a standard force of 70,000 gravity and a fixed period of three minutes, no displacement of pigment was ever observed for contracted melanophores.[4] But an easily discernible pigment displacement (see figure 2) was always obtained under the same conditions with expanded specimens. Moreover, essentially the same results were obtained regardless of the agency used to induce contraction (KCl and adrenalin solutions), or expansion (NaCl, acetyl choline,[5] and physostigmine[6] solutions).

Watching the melanin granules redistribute themselves after they have been displaced centrifugally, also provides an indication that the protoplasm is in a relative state of sol when the melanophores are expanded. A complete redistribution of the granules, after they are displaced as in figure 2, appears to depend upon Brownian movement. At any rate an exceedingly active Brownian movement can clearly be seen as the melanin granules begin to invade the part of the protoplasm which previously was cleared of the pigment. This movement continues for about an hour, at which time the redistribution is almost complete.

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[3] Cordial thanks are extended to Dr. E. Newton Harvey and to Dr. Ethel Brown Harvey for their kindness in permitting the use of this equipment at the Marine Biological Laboratory.

[4] In contracted melanophores no sign of pigment displacement was obtained with even greater forces (up to 125,000 gravity) employed for periods up to 12 mins.

[5] Acetyl choline chloride, Merck, 1×10^{-4}, in N/10 NaCl.

[6] Physostigmine sulfate, Merck, 3×10^{-6}, in N/10 NaCl.

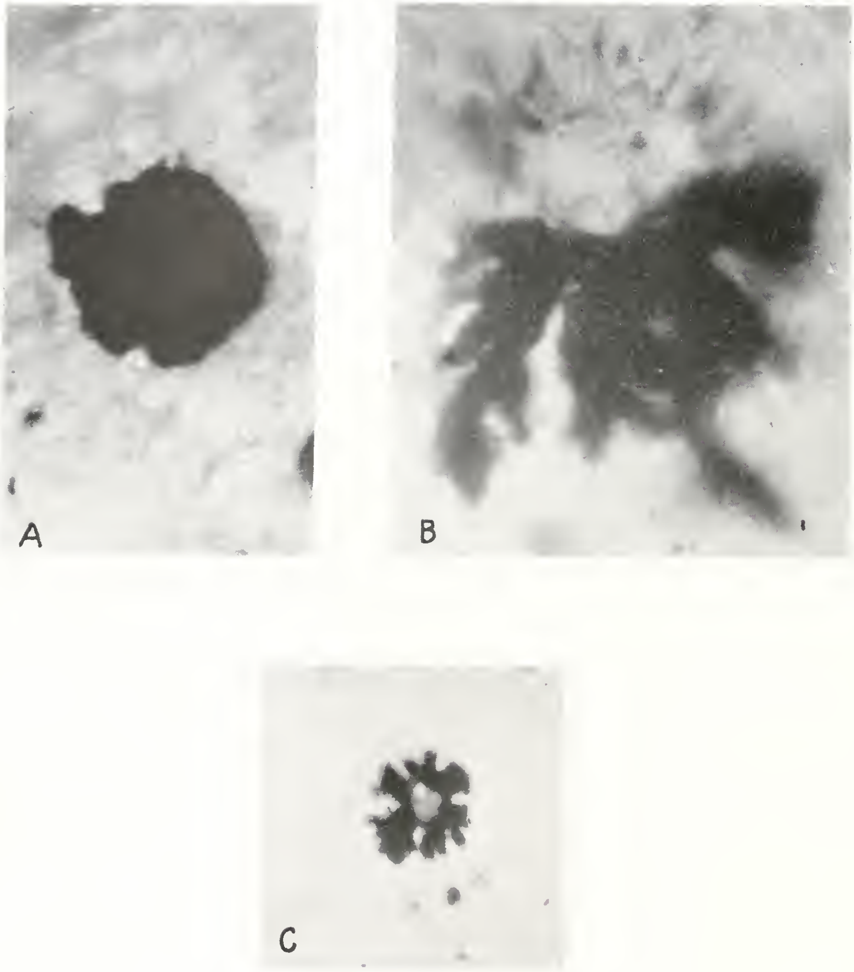

FIGURE 2. (*A*) contracted, and (*B*) expanded melanophores, both centrifuged for 6 minutes at a force of 70,000 gravity. No pigment displacement was ever obtained in contracted specimens, using forces up to 125,000 gravity. (*C*) semi-expanded specimen, showing the central hyaline plasmasol region.

Despite the drastic centrifugal treatment, the melanophores do not appear to be damaged. After the melanin granules have been redistributed and the normal expanded form has been regained, the melanophores are susceptible to further contractions and expansions, if the scales are successively immersed in solutions of KCl and NaCl.

Temperature experiments

Since it is known that the magnitude and even the sign of the pressure effect upon certain physiological processes (bioluminescence, Brown, Johnson and Marsland, 1942; and muscular contraction, Brown, 1934) depend upon temperature, some of the experiments were repeated at low (6° C.) and high (30° C.) temperatures.

These latter experiments demonstrate that high temperature fosters a contraction of the melanophores; whereas low temperature tends to induce expansion. At room temperature (20–22° C.), melanophores immersed in N/10 NaCl are all expanded; but at 30° C. they all reach a full state of contraction. Moreover, at room temperature melanophores in N/10 KCl are fully constricted; but at 6° C., a majority on a given scale are about two-thirds expanded, and the others are fully expanded. Also at 30°, when fully contracted KCl-immersed specimens are subjected to hydrostatic compression, it is very difficult to initiate expansion. In fact, the first sign of this effect does not appear until the pressure reaches about 6000/in.^{2}, in contrast to the room temperature level, namely 1000–2000 lbs./in.^{2}. At 30° C., however, the viability of the melanophores is limited to about two hrs., at least in solutions containing only NaCl or KCl. After this time irritability is lost rapidly, and no further contractions or expansions can be elicited.

DISCUSSION

The action of pressure is localized in the melanophore itself, rather than in the surviving nerve supply, as is clearly established by the work on denervated specimens. Thus it seems likely that pressure has its main effect upon some intrinsic component in the protoplasmic system.

Recent studies on the biological effects of pressure indicate that pressure exerts at least two main types of action in protoplasmic systems. The first type of action appears to be primarily chemical, in that the pressure modifies the velocity or equilibrium of one or more of the metabolic reactions which energize the physiological process; but the second effect is physical, in that pressure appears to change the viscous and tensile properties of specific gel structures in the cell.

No doubt both types of pressure effects are present in any given system, but the first kind of action seems to dominate in the studies on muscle, nerve, and luminescence; whereas the second is dominant in amoeboid movement, cleavage, and cyclosis (Marsland, 1942).

The present evidence indicates that the action of pressure upon melanophores is mainly of the second type, and that sol-gel changes are definitely concerned with the development of the forces which cause the granules to flow back and forth in the protoplasmic branches of the pigment cells. However, quantitative measurements to substantiate this view could not be obtained, and consequently the qualitative evidence must be considered very carefully.

Pressure determines the dispersion and concentration of the melanophore pigment in a very regular and decisive fashion, and this action is clearly parallel to the pressure effects upon amoeboid movement, cleavage, and cyclosis. This leads to the conclusion that sol-gel changes are likewise of critical importance in melanophore activity. According to this view, contraction of the melanophore is determined by a gelation of the protoplasm; whereas expansion depends upon solation.

This hypothesis permits a logical interpretation of the observed effects of both temperature and pressure. The gel system of the melanophore clearly conforms to that of the several other protoplasmic gels which have been studied, in that it behaves like a type III gel (Freundlich, 1937). This type of gel *sets more firmly with increasing temperature* and, in setting, undergoes a small but *definite increase of volume*. Type III gels invariably undergo solation when the pressure is increased, or when the temperature is lowered (Marsland and Brown, 1942). Consequently the facts: that both high pressure and low temperature regularly cause expansion in the melanophore; and conversely, that decreasing pressure and increasing temperature regularly bring about contraction, may be considered as strong evidence in favor of the hypothesis.

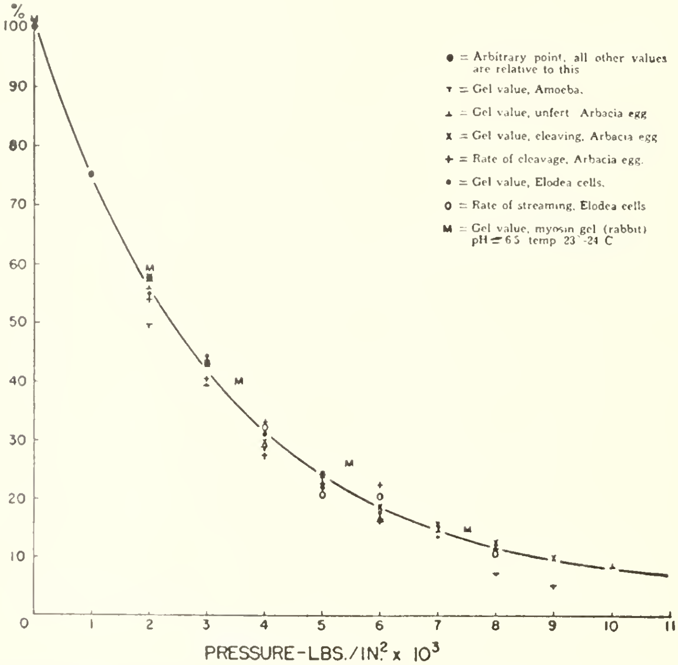

FIGURE 3. Inhibition of protoplasmic movements in relation to the solating effects of hydrostatic pressure.

A substantiation of this view must await more data, but the existing evidence seems very significant. The contraction phase of the pigmentary response is limited by pressure in a manner that parallels, at least roughly, the inhibition of gelation which has been demonstrated in plasmagel systems generally. The "half-expanded" state of the chromatophore (Fig. 1) occurs at about 2500 lbs./in.² (at 20°–22° C.), which corresponds to a gelation value of approximately 50 per cent (Fig. 3); and it seems probable that the other values, should they become available, will likewise fall upon the general curve. Also the centrifuging experiments provide at least a qualitative demonstration that gelation and solation are of critical significance in melanophore activity. Regardless of the agent (NaCl, physostigmine and acetylcholine solutions) which was used to bring about the pigment dispersal, expanded melanophores always showed a clear displacement of the melanin granules at a centrifugal force of 70,000×g.; whereas this force never gave any sign of pigment displacement in contracted melanophores.

It seems worthwhile to speculate briefly as to how gelation may instrument the movement of the pigment from branches into the body of the chromatophore. The simplest concept of the mechanism comes from the views of Lewis, 1942, and Marsland, 1942, which were derived from studies on amoeboid movement and cleavage. According to this view every gel, by virtue of its intrinsic structure, tends to contract spontaneously. The gel is conceived of as a colloidal network of interconnected protein units, the interstices of which are filled with a fluid residuum of the original sol. Conditions which foster gelation lead to a strengthening of the interconnecting bonds and to a folding of the extended protein units which constitute the framework of the gel. These factors account for the contractile tendency, which is accompanied by an exudation of sol, expressed from the shrinking interstitial spaces.

In the melanophore, the pigment granules are probably enmeshed in, or attached to, the gel framework which, in the expanded specimen, extends out into the outlying branches. During contraction, conditions favoring a firmer setting of the gel are imposed upon the system and consequently the gel framework begins to shrink and pull the pigment granules inward from the extended branches. Simultaneously, however, these must be an outflow of sol into the branches, in sufficient quantity to compensate for the volume of the retreating gel and pigment.

Direct observations of the melanophores under oil immersion tends to support the foregoing hypothesis. In the partially and fully expanded specimen the central region of the body of the melanophore is clearly hyaline, and the pigment granules are located only in the peripheral parts of the body and in the extended branches (Fig. 2). This differentiation indicates that the plasmasol and plasmagel of the melanophore occupy the same relative positions in amoeboid cells generally. Consequently it is proposed that the hyaline protoplasm of the body of the melanophore be considered as the *plasmasol,* which is surrounded by the pigmented outlying protoplasm, the *plasmagel.*

During contraction the central hyaline plasmasol region of the melanophore becomes obliterated. No doubt this results from the encroachment of pigment granules which come in from the branches. But in the process of this obliteration, probably, the framework of the plasmagel must shrink, exerting a pressure upon the enclosed sol. Thus part at least of the sol must seep out through the meshes of the surrounding gel and escape into the branches to replace the material which is retreating from these parts. Such an exudation of hyaline sol through the meshwork of the surrounding gel would be homologous to the escape of sol which occurs through the plasmagel sheet at the advancing tip of a pseudopodium in Amoeba (Mast, 1926).

The outlying branches of the melanophore persist during contraction (Matthews, 1931). But no one has been able to observe the outflow of hyaline sol, which must inevitably occur while the pigment is retreating from the branches. The difficulties of such observations are, no doubt, first that the sol is completely hyaline, and second, that the branches tend to be obscured by other tissues occupying a more superficial position on the scale (Matthews, 1931).

Indirect evidence of the outflow of sol may be obtained by observing (oil immersion) the inflow of pigment granules from the peripheral tips of the branches of the melanophore during the early stages of contraction. These granules behave as if they were being dragged, so to speak, against the stream. They exhibit a peculiar bobbing movement which is distinctly different from Brownian movement. The individual granules tend to arrange themselves on a linear series and do not change their relative positions despite the irregularity of their movement. Later, when contraction nears completion and the pigment reaches the stouter trunk-like origin of each branch, the linear arrangement of the granules is even more accentuated, but the bobbing movements have practically subsided. These observations appear to reinforce two main points in the sol-gel hypothesis: first that the pigment granules are definitely affixed in the contracting gel framework and consequently tend to display a definite pattern of arrangement; and second, that the contraction of the gel framework generates an outward flow of the hyaline sol, derived partly from the central fund of plasmasol and partly from the interstices of the gel itself.

No evidence can be offered as to the mechanism of relaxation, which redistributes the pigment granules after a contraction has abated, except that the protoplasm of the pigment cells always shows a definite degree of solation when relaxation occurs. Compared to the very firm gelation of the contracted state, this solation may be just as great as the solations which have been demonstrated in such relatively loose gels as the plasmagel of the amoeba. But the whole system of the melanophore is pitched at a higher level of gelation. This is indicated by the great centrifugal force necessary to displace the pigment even in the relaxed cells. In the amoeba, a force of less than 7000 gravity is adequate to displace all granules even when the plasmagel is set to its maximum firmness, but in the melanophore a ten times greater force (70,000) is needed, even when the gel system is at minimum "solidity." Apparently there is a very definite residuum of gel structure in the melanophore protoplasm even under conditions of maximum solation. Consequently it is possible that relaxation results from an unfolding of this persistant gel mesh-work, by a reversal of the same processes which determine its contractile folding. In any event it is plain that redistribution of the pigment does not depend on Brownian movement. In drastically centrifuged melanophores, in which presumably the pigment granules have been torn loose from their connection with the gel structure, more than an hour elapses before the displaced granules reach the periphery of the cleared protoplasm in the body and branches of the pigment cells.

SUMMARY

(1) Increasing hydrostatic pressure progressively inhibits the concentration of melanophore pigment, at least roughly in proportion to the magnitude of the pressure, in the range up to 7000 pounds per square inch. At each higher pressure the capacity to contract is further reduced, not only in the case of pulsating melanophores (Spaeth method), but also in the case of steady contractions induced by various chemical agents.

(2) This action of pressure is entirely independent of the nerve supply of the melanophores, since denervation does not in any way alter the pressure responses of the pigment cells.

(3) Low temperature (6° C.) reinforces the pressure inhibition of contraction, but high temperature (30° C.) has a counteracting effect.

(4) Both the pressure and the temperature effects indicate that contraction depends upon the capacity of the protoplasm of the pigment cells to undergo gelation; whereas expansion involves solation. This hypothesis, which is borne out by a number of microscopic observations, brings melanophore activity into line with several other types of protoplasmic movement.

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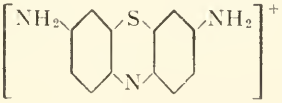
ERRATUM

In the October 1944 issue in the article "The theory of metachromatic staining" by L. Michaelis the formula of thionin on page 156 was incorrectly stated and should read as follows:



formula of thionin
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neutral or slightly acid solution)

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